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Dissertation

Investigation of *cis* acting elements and *trans* acting factors in the promoter of the xylanase I gene of *Trichoderma reesei (Hypocrea jecorina)*

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Kurzfassung

Der industriell eingesetzte (Buchert, Oksanen *et al.* 1998; Galante, De Conti *et al.* 1998; Galante, De Conti *et al.* 1998) Weichfäulepilz *Trichoderma reesei* (der Anamorph von *Hypocrea jecorina*) is ein höchst effizienter Produzent von holzabbauenden Enzymen, wobei das enzymatische Spektrum Cellulasen (CBH I, CBH II, CBH III), β-Glucosidasen (BGL I, BGL II), Endoglucanasen (EG I, EG II, EG III, EG V, EG VII), Xylanasen (XYN I, XYN II) und einige seitenkettenabbauende Enzyme (ABF I, GLR I, AXE I, AE) umfaßt. Der Pilz sezerniert bei Induktion des Cellulase-Systems bis zu 60 g/l Protein, wovon 70% Cellulasen sind. Unter diesen ist das vorherrschende Protein CBH I (ungefähr 60% (Fowler, Grizaldi *et al.* 1993)).

Daher wurde der Regulation der Proteinproduktion in diesem Pilz einiges an Aufmerksamkeit zuteil. Dennoch ist bis heute relativ wenig darüber bekannt, wie die Produktion extrazellulären Proteins kontrolliert wird. Nur vier Gene (*cbh1*, *cbh2*, *xyn1*, *xyn2*) wurden detaillierter dahingehend untersucht, was sich bei ihnen auf molekularer Ebene abspielt (Stangl, Gruber *et al.* 1993; Ilmén, Onnela *et al.* 1996; Mach, Strauß *et al.* 1996; Zeilinger, Mach *et al.* 1996; Zeilinger, Mach *et al.* 1998; Zeilinger and Mach 1998; Zeilinger, Haller *et al.* 2000; Saloheimo, Aro *et al.* 2000; Aro, Saloheimo *et al.* 2001). Für diese depolymerisierenden Enzyme wurde gezeigt, daß die Kontrolle ihrer Synthese auf Transkriptionsebene stattfindet (Shoemaker, Schweickart *et al.* 1983; Teeri, Salovouri *et al.* 1983; Teeri, Lehtovaara *et al.* 1987; Penttilä, Lehtovaara *et al.* 1986; Saloheimo, Lehtovaara *et al.* 1988; El-Gogary, Leite *et al.* 1989; Messner and Kubicek 1991; Fowler and Brown 1992; Morawetz, Gruber *et al.* 1992; Penttilä, Saloheimo *et al.* 1993; Ilmén, Onnela *et al.* 1996; Mach, Strauß *et al.* 1996; Zeilinger, Mach *et al.* 1996; Zeilinger and Mach 1998; Zeilinger, Mach *et al.* 1998; Zeilinger, Haller *et al.* 2000; Margolles-Clark, Ihnen *et al.* 1997; Würleitner, Pera *et al.* 2002).

Faktoren, für die gefunden wurde, daß sie die expression dieser Gene steuern und die auch kloniert wurden, sind Cre1 (Ihnen, Thrane *et al.* 1996), ACE I (Saloheimo, Aro *et al.* 2000), ACE I (Aro, Saloheimo *et al.* 2001) und der HAP 2/3/5-Komplex (Zeilinger, Ebner *et al.* 2001). Crel und ACE I wirken als Repressoren der Transkription (Mach, Strauß *et al.* 1996; Aro, Ilmén *et al.* 2003; Takashima, Iikura *et al.* 1996; Ilmén, Onnela *et al.* 1996; Strauß, Mach *et al.* 1995), wohingegen ACE II und in einigen Fällen auch der HAP 2/3/5-Komplex

die Genexpression ankurbeln (Aro, Saloheimo *et al.* 2001; Aro, Ilmén *et al.* 2003; Zeilinger, Mach *et al.* 1998; Zeilinger, Ebner *et al.* 2001). In anderen Fällen kann der HAP 2/3/5-Komplex auch die Transkriptionsaktivität reduzieren (Würleitner, Pera *et al.* 2002). Die unterschiedlichen Effekte, die aus der Bindung von HAP 2/3/5 resultieren könnten auch mit der jeweiligen Gesamtstruktur des Promotors und dem Abstand der CCAAT-Box zum Transkriptionsstart zusammenhängen, da gezeigt wurde, daß der Komplex die Positionierung von Nucleosomen beeinflußt (Narendja, Davis *et al.* 1999).

In dieser vorliegenden Arbeit wird ein weiterer neuer Transkriptionsaktivator präsentiert, der Xyrl benannt wurde, und der ein $(Zn^{2+})_2 \cdot Cys_6$ Zink-Cluster Protein ist, das zur Familie der GAL4-Faktoren gehört. Xyrl ist homolog (47,2% Identität) zum Xylanase-Regulator XlnR aus *Aspergillus niger* (van Peij, Visser *et al.* 1998) und unverzichtbar für ein erhöhtes Transkriptionsniveau des *xyn1*-Gens aus *T. reesei* bei Induktion. Das entsprechende Gen wurde kloniert und im Detail analysiert.

Auch die Effekte der *cis*-agierenden Elemente im *xyn1*-Promotor wurden in dieser Arbeit *in vivo* studiert, wobei zum ersten Mal der Promotor in voller Länge verwendet wurde. Zwei Motive waren bereits früher als Regulatoren der Expression dieses Gens identifiziert worden. Eine Cre1-Bindungsstelle, die für Kohlenstoff-Katabolit-Repression verantwortlich ist (Mach, Strauß *et al.* 1996) und eine CCAAT-Box, die den HAP 2/3/5-Komplex bindet (Zeilinger, Mach *et al.* 1996; Rauscher, Würleitner *et al.*, Manuskript in Vorbereitung). Für ein drittes Element mit der Konsensus-Sequenz der *A. niger* XlnR-Bindungsstelle (GGCTAA), hier vorhanden als inverse Wiederholung, wurde ebenfalls eine Rolle im Regulationsprozeß vermutet (Wacenovsky 1998). Indem ein Reportergenkonstrukt angefertigt wurde, das den *xyn1*-Promotor vor dem Glucoseoxidase-Gen (*goxA*) aus *A. niger* enthält und durch das Einfuhren von spezifischen Mutationen in den einzelnen Elementen (XLNR, CCAAT, CRE) im Promotor konnte gezeigt werden, daß das der XlnR-bindenden Stelle ähnelnde Element (XLNR) und sein korrespondierender *trans*-agierender Faktor (der mit höchster Wahrscheinlichkeit Xyrl ist), essentiell für die Induktion der Expression von XYN I *in vivo* ist und es konnte bestätigt werden, daß die Kohlenstoff-Katabolit-Repression durch die doppelte Cre1-Stelle (CRE) vermittelt wird. Ebenso konnte bewiesen werden, daß die CCAAT-Box ebenfalls an der Regulation beteiligt ist, wenn sie auch alleine das Transkriptionsniveau nicht signifikant beeinflussen kann.

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Theoretical Part

Introduction

In World War II U.S. forces stationed on the picturesque island Bougainville in the South Pacific encountered a severe problem concerning the part of their equipment which was made of cotton (mainly uniforms and tents), which was degraded rapidly in the moist and warm tropical climate. Investigations were launched and the culprit was tracked down. It was found to be a fungus belonging to the genus *Trichoderma*. Elwyn T. Reese and Mary Mandels isolated a strain named QM6a, which was first thought to be a variety of the species *longibrachiatum*, but was later found to be rather a separate species, which was then called *reesei*.

Trichoderma reesei itself is not capable of sexual reproduction, although under certain conditions a fusion of nuclei can be observed. Therefore the scientific community did not stop looking for relatives of *T. reesei* exhibiting a fully functional sexual reproduction cycle (a so called teleomorph), and nowadays we know *T. reesei* to be an asexual form (anamorph) of *Hypocrea jecorina*, as which it is used to be referred to today (Domsch, Gams *et al.* 1980).

Fig. 1: View of Bougainville

Trichoderma reesei (Hypocrea jecorina)

General Aspects

Since the discovery of the fungus *Trichoderma* species have received more and more attention. Today there is a great variety of industrial applications (Buchert, Oksanen *et al.* 1998; Galante, De Conti *et al.* 1998; Galante, De Conti *et al.* 1998) of this organism: production of extracellular enzymes (e.g. cellulases, xylanases), transformation of a wide range of complex substrates (of natural as well as of xenobiotic origin), use as a biocontrol agent of plant fungal diseases and production of a vast spectrum of secondary metabolites (e.g. antibiotics). Last but not least it serves as a model organism for the examination of the genetics, metabolism and physiology of industrially important filamentous fungi. Advantages regarding the cultivation are minimal nutritional requirements, rapid growth and the ability to conidiate profusely. Due to the fact that *Trichoderma* has uninucleate conidia, mutational analysis and screening of clones is relatively simpel, although leaky auxotrophs are common, so that double auxotrophs are preferred (Picataggio, Schamhart *et al.* 1983).

History

Trichoderma was first described by Persoon in 1794 as an asexually reproducing fungus. The Tulasne brothers revealed in 1865 (Tulasne and Tulasne 1865) the relationship between the anamorph *T. viride* and the teleomorph *Hypocrea rufa*. It has to be noted, that up to now it is very difficult to assign anamorphic species to their corresponding teleomorphs, because different teleomorphic genera can exhibit morphologically indistinguishable anamorphs (so there are *Trichoderma* species that correspond to *Hypocrea* and others that correspond to *Podostroma* teleomorphs) and a single teleomorph can also have different anamorphs (so *Hypocrea* species have predominantely *Trichoderma* anamorphs, but some species are genotypically different and possess *Gliocladium* and *Verticillium* anamorphs). Only in recent times the development of a taxonomy based on genetic data has started to implement a reliable tool to solve this puzzle.

Occurence

Trichoderma species are fast-growing hyphomycetes that show a widespread occurrence and are extremely common in agricultural, prairie, forest, salt marsh and desert soils in all climatic zones (Danielson and Davey 1973; Domsch, Gams *et al.* 1980). In particular they can

be found in the litter of humid, mixed hardwood forests, where they represent up to 3% of the fungal population, and about 1,5% in pasture soils (Brewer, Calder *et al.* 1971).

This widespread occurrence is due to their metabolic versatility, their resistance to microbial and xenobiotic growth inhibitors, the relative insensitivity of the germination of their spores to fungistasis (Emmatty and Green 1966) and their antagonism to other microbes, which provides *Trichoderma* species with a high colonisation potential. So for instance a dominance of *Trichoderma* spp. in soil following fumigation can often be found.

Taxonomy

Trichoderma (anamorph)	*Hypocrea* (teleomorph)
Deuteromycotina / Hyphomycetes	Ascomycotina / Pyrenomycetes / Hypocreales

Morphology

Growing on solid media *Trichoderma* colonies show a smooth white surface which is becoming compact following conidiation. The mycelium of some strains may be coloured yellow by secondary metabolites, but the mature colonies are green from the colour of the conidial masses. Conidia (phialospores) form on branched aerial conidiophores and are produced inside flask-shaped phialides, from where they are released at the tip. This means a basipetal succession via production of enteroblastic conidia, where the cell wall of the phialide is ruptured by the first conidium (Hammill 1974).

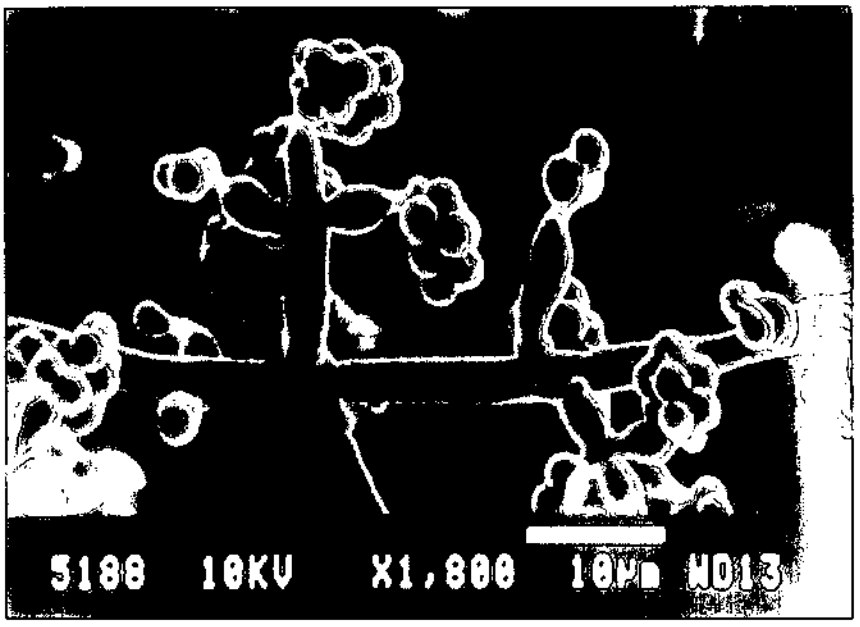


Fig. 2: Electron micrograph of *Trichoderma viride*

Nutrition and Metabolism

Trichoderma can use a wide range of substrates. Preferred polymeric carbon sources are cellulose, chitin, alminaran, pectin, starch and xylan (Danielson and Davey 1973; Domsch, Gams *et al.* 1980). Some species can utilize inulin, melezitose, raffinose, sucrose and tannic and gallic acids as well (Danielson and Davey 1973). As for nitrogen, ammonium compounds, L-alanine, L-aspartate, L-glutamic acid and proteins are a good source, although nitrate assimilation is often poor and species dependent (Danielson and Davey 1973).

It is noteworthy that *Trichoderma* species are one of the few groups of organisms that can utilize C_1 compounds like for instance methanol (Tye and Willetts 1977), and that there are also strains which can degrade hydrocarbons (Davies and Westlake 1979), so that *Trichoderma* is often found to be a major component of populations from soil polluted with oil (Gudin and Chater 1977; Llanos and Kjoller 1976; Pinholt, Struwe *et al.* 1979).

Also a high level of resistance to fungicides due to metabolic conversion of the agent can be stated. Many chemically different pesticides, such as allyl alcohol (Woodcock 1971), Arachlor (Cahal, Bans *et al.* 1976), DDT, Aldrin, Dieldrin (Matsumura and Boush 1971), Malathion, (Matsumura and Boush 1966) Dalapon (Senior, Bull *et al.* 1976) and also *Aspergillus* aflatoxin (Mann and Rehm 1976) can be transformed by the fungus.

Since *Trichoderma* has the ability to utilize such a variety of substrates and to survive under relatively adverse conditions, it can appear as a general spoilage organism as well. *Trichoderma* spp. have been detected to take part in the deterioration of paintings, masonry, rubber, plasticizers, polyethylens (Pitt 1981; Rose 1981) and even jet fuels (Sheridan 1974).

Secondary metabolites

Many different secondary metabolites are produced by the fungus. First there are a lot of mycotoxins, which can be divided by and large into three groups corresponding to different substance classes: trichothecenes, cyclic peptides and isocyanide containing metabolites.

Trichothecenes (e.g. trichodermin (Abrahamsson and Nielsson 1966)) are toxic to eucaryotes. This toxicity ist due to the fact that these substances inhibit the action of (eucaryotic) peptidyl transferase (Carrasco, Barbacid *et al.* 1973; Cutler and LeFiles 1978). They can as well provide templates for medicinals and plant growth inhibitors.

Cyclic peptides are lipophilic proteins which intercalate within the phospholipid membranes and thereby modify their ionic permeability and also promote lysis. Some of the mycotoxins of this class are used as antibiotic (e.g. alamethicin (Jung and Dubischar 1975; Meyer and Reusser 1967)).

The third group consists of isocyanides. Most of these substances are rather instable. One exception to this rule is trichovoridin, which is fairly stable (Nobuhara, Tazima *et al.* 1976). Especially *T. hamatum*, which occurs in the soil of certain sheep pastures often produces isocyanides, which markedly inhibit cellulolytic rumen microbes. This led to the assumption that the fungus participates in promoting ill-thrift of sheep (Brewer, Calder *et al.* 1971).

Then there is a large group of anthroquinone pigments which mostly show a yellow colour (e.g. pachybasin, chrysophanol, emodin (Slater, Haskins *et al.* 1967; Jenssen 1970)) and to which no function could be assigned up to now.

Last but not least there is a pool of chemically different mostly inhibitory metabolites, which are partly volatile and aid the colonisation of soil (Dennis and Webster 1971). One volatile component is 6-pentyl-α-pyrone which contributes to the characteristic coconut odour of the producing strains (Collins and Halim 1972; Kikuchi, Mimura *et al.* 1974; Moss, Jackson *et al.* 1975).

Physiology

Trichoderma mycelium cell walls comprise of β-glucans and chitin (Benitez, Villa *et al.* 1975), where chitin synthesis occurs at the hyphal tip (Mirelman, Galun *et al.* 1975). In contrast, conidia have a composition of β-1,6-glucans (~35%), β-1,3-glucans (~10%) and melanins (~21%) and do not contain any chitin (Benitez, Villa *et al.* 1976). For the onset of the conidiation a short exposure to light is necessary (Gressel, Strausbauch *et al.* 1971). In this context an analogy to the blue light receptor system of higher plants is assumed (Gressel, Strausbauch *et al.* 1971). Furthermore conidiation seems to be coupled to *de novo* synthesis of RNA (Galun and Gressel 1966; Betina and Zajakova 1978).

In the course of hyphal elongation ribosomes are not transported to the tip but produced on the spot, where protein synthesis is maximal (Stavy, Stavy *et al.* 1970).

Trichoderma species are in general excellent producers of enzymes, some strains are able to secrete up to 60 g/l of extracellular protein, 70% of which is cellulase.

Applications

Trichoderma species are already involved in many industrial processes. The fungus itself can be used as a biocontrol agent for fungal infection (Bliss 1951), because it inhibits the growth of other fungi (e.g. *Armillaria mellea*, *Heterobasidion annosum*, *Rhizoctonia solan?)* by several means. Also the effectiveness of *T. polysporum* against dry bubble disease (*Verticillium fungicola*) of mushrooms (*Agaricus* sp.) has been shown (Ricard 1977) and due

to its resistance to growth inhibitors it is helpful as a control organism in the development and evaluation of fungicides.

Furthermore this genus produces a manyfold of enzymes (cellulases, xylanases, glucanases etc.) for a broad range of applications, and there are strong promoters in the genome that allow the production of heterologuous proteins in a sufficient amount and concentration as well. *Trichoderma* enzyme preparations are used in pulp and paper industry (Buchert, Oksanen *et al.* 1998), food industry (Galante, De Conti *et al.* 1998), leather and textile industry (Galante, De Conti *et al.* 1998). In Japan cellulase preparations from *T. viride* are even used as digestive aid in geriatric food. Cell wall lytic enzymes from this fungus are taken for the formation of protoplasts from fungi (Wessels and Sietsma 1979) and plants (Evans and Bravo 1983), and *T. polysporum* preparations are registered as fungicides for the prevention of mushroom diseases (LD_{50} = 4g/kg in rats) (Ricard 1976).

Also among the secondary metabolites there are many valuable substances. Trichothecenes can be used as regulator for plant growth and as templates for medicinals (Cutler and LeFiles 1978). Some of the cyclic peptides of *Trichoderma* are already admitted for application as an antibiotic (e.g. alamethicin).

Risks

No imminent danger emanates from *Trichoderma,* although in some cases it can be a nuisance - the growth of shiitake mushrooms (*Lentinus edodes*) in industrial cultures can be inhibited by an infection with *T. viride* (Komatsu and Inada 1969), isocyanide metabolites (mainly of *T. hamatum*) are considered to promote ill-thrift of sheep (Brewer, Calder *et al.* 1971) and *Trichoderma* species in general often play a significant role in the biodeterioration of various materials and are considered as spoilage organisms (Pitt 1981; Rose 1981). The hazard to humans is very low: only one well-defined instance of pathogenity is reported (Loeppky, Sprouse *et al.* 1983).

The polysaccharide degrading enzymes of *T. reesei*

As a soft rot fungus, *Trichoderma* is able to grow on plant polysaccharides. The most abundant among these is cellulose, chemically a polymeric β-1,4-D-glucopyranoside of rather high crystallinity. In plant cell walls cellulose is accompanied by other structural polymers, the most prevalent (20-35%) ones belonging to the group of hemicelluloses (Eriksson, Blanchette *et al.* 1990).

Hemicelluloses are heteropolysaccharides composed of two or more monosaccharides such as D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose and 4-O-methyl-D-glucuronic acid linked by a ß-l,4-bond to a polymeric backbone. The degree of polymerisation is with about 200 much lower than in case of cellulose. The hydroxyl residues of the monomers in the chain may additionally be esterified with acetic, ferulic and p-coumaric acids.

According to the monomers forming the backbone the hemicelluloses are classified as xylans, mannans or glucomannans. Additionally hemicelluloses carry various side-chain substituents of different sugars or sugar derivatives.

In hardwood for instance a major hemicellulase component is O-acetyl-4-O-methyl-D-glucurono-D-xylan and a minor component is glucomannan (Timell 1967; Wilkie 1983). A major softwood hemicellulose is O-acetyl-D-galacto-D-gluco-D-mannan, the second most abundant is L-arabino-D-glucurono-D-xylan which in contrast to hardwood xylan is not acetylated. In cereals and grasses the most abundant hemicelluloses are arabinoxylans (Wilkie 1979).

As those polymeric substrates are insoluble and cannot enter a cell they have to be broken into much smaller parts first before they can be taken up and metabolised. Therefore organisms have to produce a variety of depolymerising enzymes and secrete them into the environment in order to be able to use plant polysaccharides as a source of nutrition. As for the complex structure of those substrates generally more than one single enzyme is needed for an efficient breakdown. Each polymeric substrate thereby requires its own set of depolymerases to be efficiently consumed by an organism.

Trichoderma species produce two major groups of polysaccharide degrading enzymes - cellulases and xylanases.

The T. reesei *cellulolytic enzymes*

According to the actual state of knowledge the cellulolytic enzyme system of *Trichoderma* comprises the following proteins:

- **Cellobiohydrolases** (E.C. 3.2.1.91):
  cellobiohydrolase I (CBH I), cellobiohydrolase II (CBH II),
  cellobiohydrolase III (CBH III)
- **Endoglucanases** (E.C. 3.2.1.4):
  endoglucanase I (EG I), endoglucanase II (EG II), endoglucanase III (EG III), endoglucanase V (EG V), endoglucanase VII (EG VII)
- **β-Glucosidases** (E.C. 3.2.1.21):
  β-glucosidase I (BGL I), β-glucosidase II (BGL II)

All these enzymes attack ß-l,4-glycosidic bonds as present in cellulose. The Cellobiohydrolases are exo-enzymes releasing cellobiose (4-O-(β-D-glucopyranosyl)-D-glucopyranose) units from the reducing (CBH I) and the non-reducing end (CBH II) of the cellulose chain. The endoglucanases cleave cellulose molecules arbitrarily somewhere within the chain producing shorter polymers and the β-glucosidases degrade cellobiose to the monomer glucose.

The enzymatic activities described above are only the prevalent functions of each group of cellulases. So some of the enzymes also have transglycosylating activities (mainly the ß-glucosidases and EG I) and depending on the substrate and its concentration (especially noted for shorter oligosaccharides) a variable cleavage pattern (star activity). Sometimes even other glycosidic bonds can be hydrolysed. For instance endoglucanases often exhibit activity towards substituted cellulose and some towards xylan (EG I).

The relation of the amount of the different cellulases formed on induction is always the same (60% CBH I, 25% CBH II, 15% EG I, 0,5% BG I) as is the amount of the respective mRNA, so co-regulation of the corresponding genes is assumed (Fowler, Grizaldi *et al.* 1993).

The T. reesei *xylanolytic enzymes*

Due to the more complex structure of the substrate the xylanolytic system of *Trichoderma* contains more different classes of enzymes covering a broader variety of activities and is considered to consist of the following protein types:

- **Endo-β-1,4-xylanases** (E.C. **3.2.1.8):**
  xylanase I (XYN I), xylanase II (XYN II)
- **β-Xylosidases** (E.C. 3.2.1.37):
  β-xylosidase I (BXL I)
- **a-Arabinofuranosidases** (E.C. **3.2.1.55):**
  α-arabinofuranosidase I (ABF I)
- **α-Glucuronidases** (E.C. 3.2.1.131):
  α-glucuronidase I (GLR I)
- **Acetylxylan esterases** (E.C. **3.1.1.72):**
  acetylxylan esterase I (AXE I)
- **Acetyl esterases:**
  acetyl esterase (AE)

About 80% of the xylanolytic activity of *T. reesei* accounts on the two endo-β-1,4-xylanases XYN I and XYN II, which are supposed to have evolved from the same ancestral gene (Törrönen, Kubicek *et al.* 1993). They have been purified (Törrönen 1992), their substrate specificity has been characterised (Biely 1993), their genes isolated (Törrönen 1992; Saareleinen 1993) and their three-dimensional structure determined (Törrönen and Rouvinen 1995; Törrönen, Harkki *et al.* 1994). They have both a low molecular mass and act very specifically (for example they do not hydrolyze cellulose), they both cleave mainly unsubstituted parts of the main chain and hydrolyse xylan to the same degree. A synergism with other xylanolytic enzymes able to remove side chains and substituents and thereby creating new sites for xylanases is observed. But there are also differences in their mode of action. They show different bond cleaveage frequencies of xylooligosaccharides (Biely, Vršanská *et al.* 1993) and when acting on polymeric xylan, the ratio of xylose to xylobiose produced is considerably higher with XYN I.

XYN I has also a greater tolerance to some substituents common in xylan and a greater catalytic versatility. It shows appreciable hydrolysis of xylobiose and hydrolyzes O-acetylglucuronoxylan to a much higher degree (Biely, Vršanská *et al.* 1993).

The β-xylosidase BXL I acts as exo-β-xylanase (β-D-xylan xylohydrolase) forming D-xylose as the only product (Herrmann, Vršanská *et al.* 1997; (Margolles-Clark, Tenkanen *et al.* 1996). The expression of BXL I can be enhanced by adding D-xylose to the medium at low pH (Kristufek, Zeilinger *et al.* 1995).

The removal of side chains makes the xylopyranosyl residues of either the xylan main chain or xylooligosaccharides more accessible to degradation by xylanase or ß-xylosidase. This synergistic action is performed mainly by two enzymes, the α-glucuronidase GLR I and the α-arabinofuranosidase ABF I.

GLR I has been purified, characterized and the corresponding gene isolated (Margolles-Clark, Saloheimo *et al.* 1996; Siika-aho, Tenkanen *et al.* 1994). It shows a strict substrate requirement acting exclusively on xylooligosaccharides which carry the 4-O-methylglucuronic acid linked to the terminal xylopyranosyl unit at the non-reducing end of the oligosaccharide (Siika-aho, Tenkanen *et al.* 1994). Therefore it is only efficient in synergy with xylanase and ß-xylosidase.

ABF I has also been purified and characterized (Poutanen 1988) and the respective gene was isolated (Margolles-Clark, Tenkanen *et al.* 1996). ABF I exhibits a wider substrate specificity than GLR I. The enzyme can remove α-1,3-linked arabinofuranosidase side groups from single substituted xylanopyranosyl units both from xylooligosaccharides and from polymeric xylan. It does not seem to attack the arabinofuranosyl residues in double substituted xylanopyranosyl units (Margolles-Clark, Tenkanen *et al.* 1996). In synergistic action with endo-β-1,4-xylanases enhanced activity on polymeric xylan can be detected (Poutanen 1988).

Some of the hydroxyl groups of the D-xylose units of xylan can frequently be esterified by acetic acid. Enzymes capable of removing these substituents are acetylxylan esterase (AXE I) and acetyl esterase (AE).

AXE I is highly active on polymeric xylan and can liberate up to 90% of the acetyl substituents (Poutanen, Sundberg *et al.* 1990). Synergism with other depolymerases is very modest and can only be found to a low extent with the endo-β-1,4-xylanases and ß-xylosidase (Tenkanen, Siika-aho *et al.* 1996). The enzyme binds specifically to cellulose. This function could be mapped to the C-terminus of the protein which contains a fungal type cellulose binding domain (CBD) separated from the catalytic core by a typical linker region. Removal

of the CBD though does not affect the activity of AXE I towards soluble or fibre bound xylan (Margolles-Clark, Tenkanen *et al.* 1996).

AE is responsible for the removal of the last 10% of acetyl substituents in acetylglucuronolacton not accessible to AXE I (Tenkanen, Siika-aho *et al.* 1996). These acetyl groups seem to be located close to 4-O-methylglucuronic side groups (Puls 1992), (Tenkanen, Siika-aho *et al.* 1996). AE shows clear activity against short oligomeric and monomeric acetates (Poutanen and Sundberg 1988; Poutanen, Sundberg *et al.* 1990), thereby exhibiting the highest activity towards xylobiose acetylated on the non-reducing xylopyranosyl residue (Poutanen, Sundberg *et al.* 1990). High synergy of AE with β-xylosidase was observed (Tenkanen and Poutanen 1992).

Enzyme activity	Enzyme	Molecular Mass	pI	pH-optimum
Endo-β-1,4-xylanase	XYN I	19 kDa	5,5	4,0-4,5
	XYN II	20 kDa	9,0	5,0-5,5
β-Xylosidase	BXL I	100 kDa	4,7	4,0
α-Arabino-furanosidase	ABF I	53 kDa	7,5	4,0
α-Glucuronidase	GLR I	91 kDa	5,0-6,2	4,5-6,0
Acetylxylan esterase	AXE I	34 kDa	6,8 ; 7,0	5,0-6,0
Acetyl esterase	AE	45 kDa	6,0 ; 6,8	5,5

Table 1: The xylanolytic enzymes of *T. reesei*

Aside from the cellulolytic and the xylanolytic system also other polysaccharide degrading enzyme systems have been found to be present in *Trichoderma*, so for instance chitinases, a-glucanases, pectinases, trehalases and β-mannanases and respective accessory enzymes necessary for an effective and thorough breakdown.

Control of gene expression in general

The term *gene expression* depicts the process from the point where a gene is activated by a signal to the point where the corresponding gene product is ready to exert its assigned function. Many of the gene products are proteins.

In the process of gene expression several steps can be discriminated:

- Gene activation
- Transcription of the gene into mRNA
- Translation of the mRNA into protein

This basic scheme describes sufficiently the procedure of gene expression in procaryotes. There the DNA is not sequestered in a nucleus but embedded directly in the cytoplasm and accessible to transcription factors and RNA polymerase. At the same time the genetic information is copied to mRNA the ribosomes start synthesising the protein according to the still elongating mRNA matrix.

Generally a gene consists of three components - the promoter, the structural gene and the terminator. The promoter is the DNA region upstream of the structural gene that controls the rate of its transcription. It contains target sequences (so called *cis* acting elements) for binding proteins (the so called *trans* acting factors) favouring or inhibiting the transcription process. The summarised effect of all factors binding to the promoter gives the final transcriptional activity of a gene. Transcription itself is carried out by RNA polymerases.

In bacteria there is mainly one RNA polymerase of rather simple structure. The *E. coli* RNA polymerase holoenzyme for instance shows a subunit composition of $\alpha_2\beta\beta'\sigma$. This holoenzyme settles at the promoter, unwinds the DNA thereby forming a so called *open complex* which has a high affinity to the DNA and finally starts copying the DNA template into RNA. As the RNA polymerase advances, it leaves its σ factor behind having only the $\alpha_2\beta\beta'$ core enzyme moving along the DNA. The a factor is not necessary for transcriptional activity but for guiding the RNA polymerase to the promoter site. It is responsible for the selection of the promoters the RNA holoenzyme can actually bind to. Each individual a factor has a different specificity for certain promoter configurations.

There are several classes of a factors present in bacteria. Two of the most important classes are the σ^5 and the σ^{70} subunits. Most of the genes of *E. coli* are transcribed by RNA polymerase holoenzymes containing a σ^{70}-class subunit. Such holoenzymes are able to activate genes without the aid of additional factors stimulating transcription, although mostly only to a low extent. The σ^7 dependent promoters are organised rather strictly. They contain a TATAAT-hexamer 10 base pairs upstream from the transcription start and a TTGACA-hexamer 35 base pairs upstream from the transcription start. The overall efficiency of transcription is defined by the whole promoter structure and can be influenced additionally by repressing or activating proteins binding to the promoter as well. The influence is exerted by protein-protein interaction, therefore the sites where such factors can bind are rather restricted regarding their position to the binding site of the RNA polymerase.

The σ^{54}-dependent promoters have much more similarity with eucaryotic promoters. RNA polymerase holoenzymes containing a σ^{54} subunit are not able to form an open complex all by themselves. To this end additional factors are needed which help unwinding the DNA by interacting with the RNA polymerase, this action being accompanied by hydrolysis of ATP. The binding sites for these transcriptional activators are located about 110 base pairs upstream of the transcription start, but can also be found further upstream. To facilitate the direct protein-protein interaction the DNA between both binding sites is bent thereby forming a loop. Also in this type of promoters additional sites can be present for other proteins enhancing (or repressing) the transcriptional activity.

The process of gene expression in eucaryotes is somewhat a little more complex. In a eucaryotic cell the genome is packed tightly in the nucleus. The eucaryotic DNA is wound around nucleosomes (a complex of basic proteins, the *histories)* forming a very compact structure, the *heterochromatin* (Wolffe 1992). This structure has to be loosened first in order to facilitate transcription (Owen-Hughes and Workman 1994; Paranjape, Kamakaka *et al.* 1994). On the whole only about 10% of the genome of a cell is in a transcriptional active state allowing RNA synthesis. The protein synthesis on the other hand takes place in the cytoplasm, so the mRNA has to be exported from the nucleus. Furhtermore eucaryotic genes are generally interrrupted by non-coding sequences, called *introns*, which have to be removed from the RNA transcript to yield mature mRNA that can be translated into protein.

So the process of gene expression in eucaryotes comprises the following stages:

- Gene activation
- Transcription of the gene into hnRNA
- Modification and splicing of the hnRNA to mRNA
- Transport of the mRNA into the cytosol
- Translation of the mRNA into protein

Influence on the final level of protein produced can be taken at each of the stages listed above. First of all, the condensed heterochromatin has to be melted and histones have to be repositioned (Steger and Workman 1996) in order to make the DNA accessible to transcription factors and RNA polymerases (Hager, Smith *et al.* 1995; Svaren and Horz 1996). Domain control regions on the DNA binding wide domain regulators are responsible for the decondensation process. The affinity of the histone complex to the DNA is modulated by the different isoforms of histone proteins present in the histone complex and by the degree of acetylation of the histone proteins (Bradbury 1992). As the DNA is charged negatively, an elevated level of acetylation (hyperacetylation) will reduce the affinity of the histone complex to DNA, so that it can dissociate off easily, whereas a reduced level of acetylation (hypoacetylation) will increase the affinity. According to this hyperacetylated histones are detected at active chromosomal loci (Hebbes and al. 1994), whereas hypoacetylated histones appear in heterochromatin regions (Braunstein and al. 1993; Jeppesen and Turner 1993; O'Neill and Turner 1995; Turner, Birley *et al.* 1992). Histone acetyl transferases (HAT) catalyse the acetylation of histone proteins thereby contributing to gene activation. HAT activity is found in many complexes shown to stimulate transcription (e.g. the ADA / SAGA complexes, P/CAF (Yang and al. 1996), TAFII250 (Mizzen and al. 1996), p300/CBP (Bannister and Kouzarides 1996), ACTR (Chen and al. 1997), SRC-1 (Spencer and al. 1997)). In agreement with these data histone deacetylase (HDAC) activity can be found in some transcriptional repressors and co-repressors (e.g. Rpd3 (Taunton, Hassig *et al.* 1996), Sin3 (Laherty and al. 1997), N-CoR/SMRT (Heinzel and al. 1997)).

Another possibility is the repositioning of nucleosomes by factors hydrolysing ATP. Such factors are for example the Swi-Snf complex (Kwon and al. 1994), Nurf (Tsukiyama and Wu 1995), CHRAC (Varga-Weisz and al. 1997), ACF (Ito and al. 1997) or RSC (Cairns and al. 1996).

When the promoter has been rendered accessible, binding *of trans* acting factors is the next step to transcription, because like in procaryotic σ^{54}-dependent promoters in most cases eucaryotic RNA polymerase cannot initiate transcription all alone, or just to a low extent. A set of additional inducing factors is necessary to reach a significant level of transcription. Several types of *trans* acting factors regarding the structure of their DNA binding domain have been identified up to now:

- Helix-turn-helix (HTH) proteins (e.g. the λ-repressor)
- Zinc-finger proteins
  - * Proteins of the Zn^{2+}·Cys_2His_2 type (e.g. TFIIIA, ACE I)
  - * Proteins of the Zn^{2+}·Cys_4 type (e.g. the GATA factors)
  - * Proteins of the $(Zn^{2+})_2$·Cys_6 type (e.g. GAL4, XlnR, ACE II)
- Steroid hormone receptors (e.g. the glucocorticoid receptor)
- Basic leucin zipper proteins (e.g. GCN4)
- Basic helix-loop-helix (bHLH) proteins (e.g. Max)
- ß-sheet proteins (e.g. $NF_\kappa B$, MetJ)

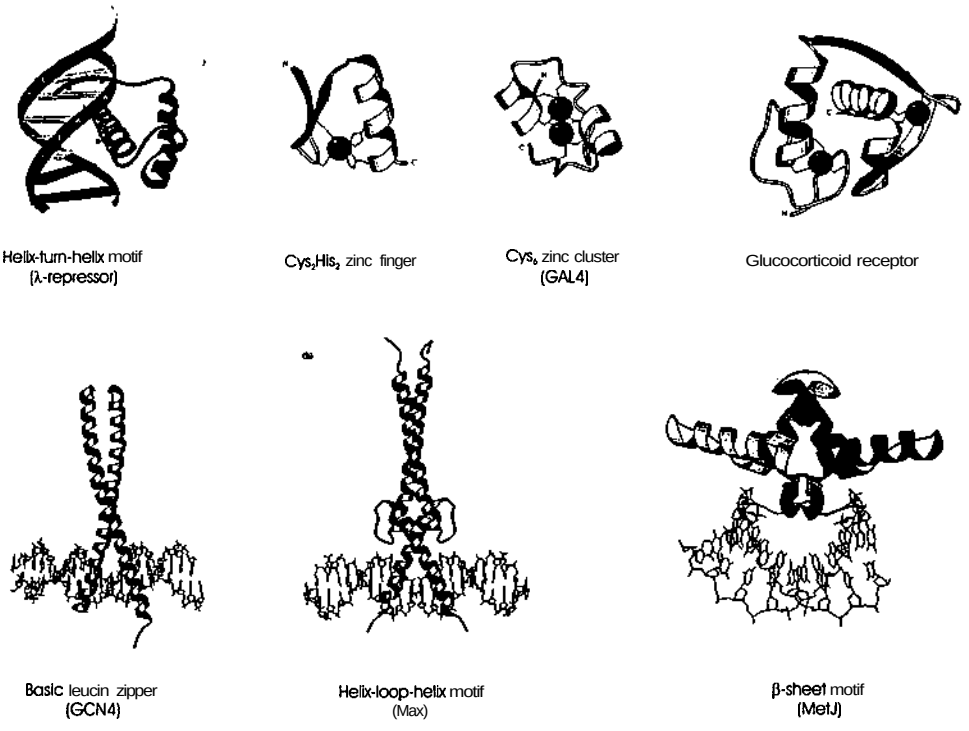


Fig. 3: Common structures of DNA binding proteins

Each *trans* acting factor binds to a specific DNA sequence in a promoter. This sequence is called the corresponding *cis* acting element. The minimum sequence requirement for binding of a factor is referred to as the *consensus sequence* of an element. Common promoter elements are:

Inr — denominates the initiator region. This is the area where RNA polymerase II (Pol II) actually binds to. Its consensus sequence generally reads Y_2CAY_2 and stretches from bp -3 to bp +5 relative to the transcription start. A promoter containing only the *Inr* element is the theoretical minimal configuration necessary for transcription by Pol II.

TATA Box — serves for the exact positioning of the translation complex, therefore also of Pol II. This element is found in many eucaryotic promoters and is recognised by the TATA binding protein (TBP), a subunit of TFIID. When no TATA box is present, transcripts of the gene show uneven 5' ends. The consensus sequence is TATAAAA and is usually situated at about bp -25 relative to the transcription start. The level of transcription normally is not affected by the presence or absence of this element. In many cases it is flanked by G/C rich sequences, which can participate in the function of the element. In absence of a TATA box such G/C rich regions serve as target for TBP but do not define an exact binding position.

CCAAT Box — works as basal transcription enhancer elevating the general transcriptional level of a gene. The position relative to the transcription start can vary considerably. Generally this element is located at about bp -80 having a consensus sequence of GGCCAATCT (Alberts, Bray *et al.* 1989). This box enhances the efficiency of a promoter but has no effect on its specificity. A lot of different factors are known to bind to this element. So for instance CTF/NF1, NF-Y, CP1, CP2, C/EBP (recognises preferably GCAAT), ACF (prefers CCAAT), CDP, CP3 (can bind to other target sequences as well) - and possibly also inducible factors. Inhibition of binding of the corresponding *trans* acting factor (either by eliminating the box from the promoter or because of a non-functional factor) can abolish gene

expression in some cases. In this context the function of CDP (*CAT-displacement protein*) is mentionable. It binds to the CCAAT box without stimulating the transcriptional activity of a gene. By this means an activator can be displaced and the gene shut off or at least reduced in its activity.

GC Box — is also a basal transcription enhancer, has the consensus sequence GGGCGG and can be found at different positions within a promoter. A factor binding to it is SP1.

Octamer motif — elevates as well the basal rate of transcription, has no fixed position within the promoter hat and is defined by the consensus sequence ATTTGCAT. It is recognised for instance by Oct-1 and Oct-2.

The CCAAT box, the GC box and the octamer motif can vary in number, position and context relative to the transcription start, they can occur in any combination as well. Furthermore the CCAAT and the GC box function in either orientation in spite of their asymmetric motif. The entirety of all of these elements present in a promoter determines the constitutive rate of transcription of the adherent gene, which often can be elevated by inducible factors binding to additional response elements. Each of the three elements can also be found in enhancers, where they are packed more tightly and in combination with a great variety of other regulatory motifs.

The conditions under which a gene is transcribed and the level of transcription is determined by the combination of promoter elements present. But also after transcription to RNA influence can be exerted on the overall level of the expression of a gene. Modulating influences affect the efficiency and the way of RNA splicing, modifications of the mRNA, the rate of transport of the mRNA into the cytosol, the initiation of translation and the stability of the mRNA. At which stage the predominant part of the regulation takes place depends on the respective gene. In recent times it turnes out more and more that all genes are regulated at least partly at the transcriptional level, although for housekeeping genes the major part of the regulation still seems to be exerted by means of mRNA stability and protein turnover, whereas inducible genes generally tend to be regulated mainly on the transcriptional level.

Control of gene expression in *T. reesei*

Although *Trichoderma* species have been receiving broad industrial interest for quite some time, still very little is known about the regulation of gene expression on the molecular level. Many genes from *Trichoderma* spp. have been cloned up to date, but only few of them have been investigated in detail concerning their regulation of expression. Among these are the cellulase genes *cbh1* and *cbh2* and the hemicellulase genes *xyn1* and *xyn2* (Stangl, Gruber *et al.* 1993; Ilmén, Onnela *et al.* 1996; Mach, Strauß *et al.* 1996; Zeilinger, Mach *et al.* 1996; Zeilinger, Mach *et al.* 1998; Zeilinger and Mach 1998; Zeilinger, Haller *et al.* 2000; Saloheimo, Aro *et al.* 2000; Aro, Saloheimo *et al.* 2001).

All four genes are inducible and regulated on the transcriptional level (Shoemaker, Schweickart *et al.* 1983; Teeri, Salovouri *et al.* 1983; Teeri, Lehtovaara *et al.* 1987; Penttilä, Lehtovaara *et al.* 1986; Saloheimo, Lehtovaara *et al.* 1988; El-Gogary, Leite *et al.* 1989; Messner and Kubicek 1991; Fowler and Brown 1992; Morawetz, Gruber *et al.* 1992; Penttilä, Saloheimo *et al.* 1993; Ilmén, Onnela *et al.* 1996; Mach, Strauß *et al.* 1996; Zeilinger, Mach *et al.* 1996; Zeilinger and Mach 1998; Zeilinger, Mach *et al.* 1998; Zeilinger, Haller *et al.* 2000; Margolles-Clark, Ilmén *et al.* 1997; Würleitner, Pera *et al.* 2002). The *cbhl* and *xynl* genes are subjected to tight carbon catabolite repression, while the *xyn2* gene shows a low basal level of transcription. The cellulase genes *cbhl* and *cbh2* are regulated coordinately, whereas the xylanase genes *xynl* and *xyn2* are not.

Regulation of the *cbhl* gene:

All elements exerting a regulatory influence on the *cbhl* gene have been found to be located within a 1,15 kb fragment upstream of the start codon. In this fragment the region upstream of bp -500 mediates glucose repression, the region downstream of bp -30 is responsible for sophorose induction (Ilmén, Onnela *et al.* 1996) and the region between bp -241 and bp -72 is needed for induction by cellulose (Henrique-Silva, El-Gogary *et al.* 1996). Motifs identified in this promoter are an inverted repeat of two Cre1 binding sites at around bp -700 which can bind Crel *in vitro* in an EMS A experiment (Takashima, Iikura *et al.* 1996), another Crel binding site at about bp –1.000, seven ACE I repressor binding sites (Saloheimo, Aro *et al.* 2000) and some ACE II activator binding sites as well (Aro, Saloheimo *et al.* 2001). Normally

the *cbh1* gene is subjected to tight carbon catabolite repression, but when the double Cre1 site is rendered non-functional by a deletion, a basal expression of *cbh1* can be observed which reaches about one tenth of the fully induced level (Takashima, Iikura *et al.* 1996). To reach the fully induced level, obviously additional activating factors are necessary. The only one identified up to date is ACE II (Aro, Saloheimo *et al.* 2001). In a Δ*ace2* strain the expression level of *cbh1* on cellulose is reduced, whereas induction by sophorose is not affected (Aro, Saloheimo *et al.* 2001), indicating distinct mechanisms for both ways of induction.

Regulation of the *cbh2* gene

In the *cbh2* gene the region extending from bp -361 to bp -70 has been shown to contain all *cis* acting elements regulating the expression (Stangl, Gruber *et al.* 1993). In this region a sequence of 5'-ATTGGGTAATA-3' referred to as the *cbh2 activating element* (CAE) was identified as being essential for induction of this gene (Zeilinger, Mach *et al.* 1998). The CAE consists of two motifs - a CCAAT box on the template strand and a GTAATA element on the coding strand. A mutation of one of the two motifs leads to a significant lower level of *cbh2* induction, whereas simultaneous mutation of both motifs leads to a complete loss of *cbh2* induction on cellulose or sophorose. The CAE is occupied by the respective *trans* acting factors (one of which has been demonstrated to be the HAP 2/3/5 complex binding to the CCAAT motif (Zeilinger, Ebner *et al.* 2001)) under all conditions tested (Zeilinger, Mach *et al.* 1998) and lies within a permanently nucleosome free region. Both motifs of the CAE are as well responsible for correct positioning of nucleosome -1 covering the TATA box under repressing conditions. Induction results in the loss of nucleosome positioning downstream of CAE thus making the TATA box accessible to RNA polymerase II (Zeilinger *et al*, manuscript submitted). Also the Crel protein is involved in the positioning of nucleosomes in the regulatory region of the *cbh2* gene. ACE II plays as well an important role in the induction process. A Δ*ace2* strain is impaired in cellulose-mediated gene expression of the *cbh2* gene but not in sophorose-mediated induction (Aro, Saloheimo *et al.* 2001).

Regulation of the *xyn1* gene

A 214 bp fragment of the *xyn1* promoter extending from bp -321 to bp -534 relative to the translation start contains all information crucial for the regulation of xylanase I gene expression (Mach, Strauß *et al.* 1996; Zeilinger, Mach *et al.* 1996). Two Crel sites arranged

as an inverted repeat have been shown to be responsible for tight carbon catabolite repression. A deletion of a few bases from the motif leads to a basal level of transcription even on carbon sources mediating catabolite repression otherwise (Mach, Strauß *et dl.* 1996). Also the Crel negative strain RUT C-30 (Ilmén, Thrane *et al.* 1996) shows a basal level of *xyn1* transcription (Mach, Strauß *et al.* 1996), which finding is further strengthening the hypothesis. Addition of xylose still leads to induction of gene expression to an elevated level, so different mechanisms for induction and repression can be assumed. Furthermore a CCAAT box and two GGCTAA elements (a sequence being identical to the *Aspergillus niger* xylanase regulator XlnR binding site (van Peij, Visser *et al.* 1998)) forming an inverted repeat are present in the *xynl* promoter. These three motifs are occupied permanently by protein under all conditions tested, as it has been proven by *in vivo* footprinting analyses (Wacenovsky 1998). The *trans* acting factor binding to the CCAAT box has lately been shown to be the HAP 2/3/5 complex and the binding of the ACE I repressor to the promoter has been demonstrated (Rauscher, Würleitner *et al,* manuscript in preparation). Its involvement in downregulating the transcriptional activity of the *xynl* gene has also been proven (Aro, Ihnen *et al.* 2003). The factor occupying the GGCTAA double site has not yet been identified.

Regulation of the *xyn2* gene

The promoter of the *xyn2* gene contains all information necessary for the regulation of gene expression within a 55 bp fragment (Zeilinger, Mach *et al.* 1996). Two factors have been identified as binding there to a sequence denominated XAE (xylanase activiating element, (Würleitner, Pera *et al.* 2002)), one being the HAP 2/3/5 complex (Zeilinger, Ebner *et al.* 2001), the other being the ACE II protein (Würleitner, Pera *et al.* 2002; Aro, Saloheimo *et al.* 2001). ACE II is essential for induction dependent as well as basal transcription, the HAP 2/3/5 complex acts as a repressor, attenuating gene expression. Deletion of the CCAAT motif in this promoter results in an increase of transcriptional activity of about 30%. The XAE is contacted by its *trans* acting factors under all conditions tested (Würleitner, Pera *et al.* 2002).

Description of the *trans* acting factors

Four of the *trans* acting factors or complexes influencing the transcriptional activity of the genes mentioned above have been cloned up to date. They comprise the two repressors Crel (Ihnen, Thrane *et al.* 1996) and ACE I (Aro, Ihnen *et al* 2003; Saloheimo, Aro *et al* 2000),

the transcriptional activator ACE II (Aro, Saloheimo *et al.* 2001) and the HAP 2/3/5 complex (Zeilinger, Ebner *et al.* 2001), which seems to be involved in facilitating or elevating basal transcription.

Cre1 is a homolog of the Mig1-repressor from yeast. It belongs to the Zn^{2+}·Cys_2His_2 type of zinc finger proteins and consists of 402 amino acids giving a molecular mass of 43,6 kDa. The consensus sequence it binds to reads 5'-SYGGRG-3'. Genes where this sequence is found as a double site forming an inverted repeat are subjected to tight carbon catabolite repression, which has been proven to be mediated by Crel (Mach, Strauß *et al* 1996; Ilmén, Onnela *et al.* 1996). When binding to single sites in the promoter, Crel takes part in the positioning of nucleosomes (Zeilinger *et al,* manuscript submitted). So it is an intriguing assumption that Crel acts in a bivalent way. As a monomer it influences the accessibility of the DNA to different factors via positioning of nucleosomes, whereas shutting off gene expression completely when binding as a dimer. The activity of Crel is regulated by phosphorylation. Binding to DNA is only observed in the phosphorylated state of the protein (Cziferszky, Mach *et al.* 2002).

ACE I has been isolated by screening a *T. reesei* cDNA library for genes able to activate transcription controlled by the *cbh1* promoter of the same fungus (Saloheimo, Aro *et al.* 2000). Later it has been shown that ACE I in fact is a repressor of transcription, for instance of the *cbhl, cbh2, egl1, egl2, xyn1* and *xyn2* genes (Aro, Ilmén *et al.* 2003). This can be explained by the fact, that the cDNA clone isolated was not full-length, comprising mainly the DNA binding domain of the protein. ACE I belongs also to the Zn^{2+}·Cys_2His_2 type of zinc finger proteins and has a size of 733 amino acids containing three zinc fingers in the DNA binding domain. The consensus sequence is 5'-AGGCAAA-3'. Dependent on the promoter context sometimes a core sequence of 5'-AGGCA-3' is reported to be sufficient to allow binding of ACE I.

ACE II has been cloned by the same approach as ACE I, screening for genes being able to activate transcription of a gene under control of the *cbhl* promoter of *T. reesei* (Aro, Saloheimo *et al.* 2001). The protein consists of 341 amino acids with a calculated molecular mass of 38 kDa. The N-terminal part has a typical zinc binuclear cluster DNA binding domain of the $(Zn^{2+})_2$·Cys_6 type. ACE II recognises a consensus of 5'-GGCTAA-3'. In *Δace2* strains a significant slower induction and lower expression level is detected for the *cbh1*, *cbh2, egl2* and *xyn2* genes, but not for the *xynl* gene (Aro, Ihnen *et al.* 2003).

The HAP 2/3/5 complex, homologs of which are present in a variety of organisms, consists of three subunits, all of which have been cloned from *T. reesei* (Zeilinger, Ebner *et al.* 2001).

HAP2 is a protein of 345 amino acids giving a calculated molecular mass of 38,4 kDa. The DNA binding domain being of the ß-sheet type, and the adjacent domain responsible for subunit interaction are highly conserved from yeast to mammals. The HAP3 protein has a size of 204 amino acids and a calculated molecular mass of 22,7 kDa. It shows a highly conserved region bearing similarities to the histone-fold motif (HFM) of the H2B family, as already demonstrated for other HAP-like CCAAT binding factors (Liberati, di Silvio *et al.* 1999; Zemzoumi, Frontini *et al.* 1999). HAP5 is 283 amino acids in size corresponding to a molecular mass of 31,4 kDa. A histone-fold motif of the H2A family can be found and as well a region homologous to a domain specific for fungi (Steidl, Papagiannopoulos *et al.* 1999) and previously identified for HAP4 interaction in other HAP5 homologs (McNabb, Xing *et al.* 1995), although no HAP4 homolog has yet been identified in filamentous fungi.

Experimental Part

Aim of the work

This work should contribute to elucidate the means by which the *xyn1* gene of *Trichoderma reesei* is regulated. Prevoius studies (Mach, Strauß *et al.* 1996; Zeilinger, Mach *et al.* 1996) have shown that the regulation of *xyn1* gene expression takes place at the transcriptional level and that a 214 bp fragment of the *xyn1* promoter located between bp -534 and bp -321 contains all sites necessary for the regulation of this gene. When this fragment is removed, the remaining truncated core promoter exhibits a basal activity under all nutritional conditions tested.

In this fragment three sites could be identified up to now, two of which already have been proven functional *in vivo*. And at the beginning of this work there have been hints that the third site is not only a putative one as well (Wacenovsky 1998).

First there is a double *cre1* site at bp -382 / bp -390 which is responsible for tight carbon catabolite repression of this gene. Normally it is occupied by Crel under repressing nutritional conditions and unoccupied otherwise, which could be proven by an *in vivo* footprinting experiment (Wacenovsky 1998). A mutation in this motif that impairs the binding of Crel to both sites results in derepression of *xyn1* to a low basal level even when there is an abundance of easily metabolisable carbon sources (e.g. glucose, glycerol) present in the medium. Induction occurs separately from carbon catabolite repression and can still be observed under this conditions as well as in Cre1 negative strains, which exhibit a comparable basal expression level of *xyn1* (Mach, Strauß *et al.* 1996).

Second there is a CCAAT box at bp –427 which is occupied permanently, the *trans* acting factors binding there presumably being the HAP 2/3/5 proteins (Mach 2002; Rauscher, Würleitner *et al.*, manuscript in preparation), which have already been shown to take part in the remodelling of the chromatin structure in other genes (Narendja, Davis *et al.* 1999).

There is another putative *cis* acting element present that resembles the XlnR binding site of *Aspergillus niger* in its sequence (van Peij, Visser *et al.* 1998) and is actually able to bind the *A. niger* XlnR protein *in vitro* (Wacenovsky 1998). Additionally it has been shown by elecrophoretic mobility shift assays (EMSA) and by *in vivo* footprinting experiments, that the XlnR like double site in this promoter ($^{-424}$**GGCTAAA**TGCGACAT**CTTAGCC**$^{-403}$) is in fact occupied by one or more *trans* acting factors. The binding could be observed under repressing as well as under inducing conditions, where the complex found under repressing conditions had a significantly lower mobility (Wacenovsky 1998).

So the aim of this work was as follows:

- Investigating the functionality of the XlnR like site in the *xyn1* promoter of *T. reesei in vivo.*
- Clarifying the role of all three *cis* acting factors present in the *xynl* promoter in induction as well as in repression.
- Cloning the regulatory factor binding to the XlnR like site in the *xynl* promoter, if this site would prove functional *in vivo.*

```
-460                                                          -401
    ctgcagcaaa tggcctcaag caactacgta aaactccatg agattgcaga tgcggcccac

-400                                                          -341
    tggaatacaa catcctccgc aagtccgaca tgaagcccct tgacttgatt ggcaggctaa
                  (CRE)                                CCAAT  XLNR
-340                                                          -281
    atgcgacatc ttagccggat gcaccccaga tctggggaac gcgccgcttg aggcccgaag
               XLNR          CRE       CRE
-280                                                          -221
    cgccgggttc gatgcattac tgccatattt cagcagttaa ctaggaccgg cttgtgtcga

-220                                                          -161
    tattgcgggt ggcgttcaat ctattccggc actcctatgc cgtttgatcc gatacctgga
                                                              (CRE)
-160                                                          -101
    ggggcgtgctt taggcaaaat gccaagcttc gaggatactg tacgagccgc tttcaacctc

-100                                                           -41
    acttgatgat gtctgagttt catcaagaga attgaagtca aagctcaaat catgatgtga

-40                                                           +20
    agaggttttg aatgtggaag aattctgcat atataaagcc ATGGAAGAAG ACGTAAAACT
                                    TATA
+21                                                           +79
    GAGACAGCAA GCTCAACTGC ATAGTATCGA CTTCAAGGAA AACACGCACA AATAACATC
```

Fig. 4: Sequence of the *xyn1* promoter. The *cis* acting elements are underlined. Elements, which have not been proven functional by now are indicated by parantheses. Bold face letters mark the beginning of the XYN I protein sequence. Sequence belonging to the mature protein is marked in grey.

Investigation of the function of the *cis* acting elements of the *xyn1* promoter *in vivo*

To study the role of the *cis* acting elements in the *xynl* promoter mutations that are known to render the respective motif void were introduced into each of the three sites, covering single mutations as well as all possible combinations of the respective mutated motifs. For being able to monitor the effects of the mutated promoter on gene expression *in vivo* a reporter gene construct was designed where the *xynl* promoter precedes the *goxA* structural gene form *Aspergillus niger,* encoding for glucose oxidase. As this enzyme is secreted into the medium and has no equivalent in *T. reesei* the level of glucose oxidase activity in the medium corresponds directly to the level of gene expression of the reporter gene construct (Würleitner, Pera *et al.* 2002).

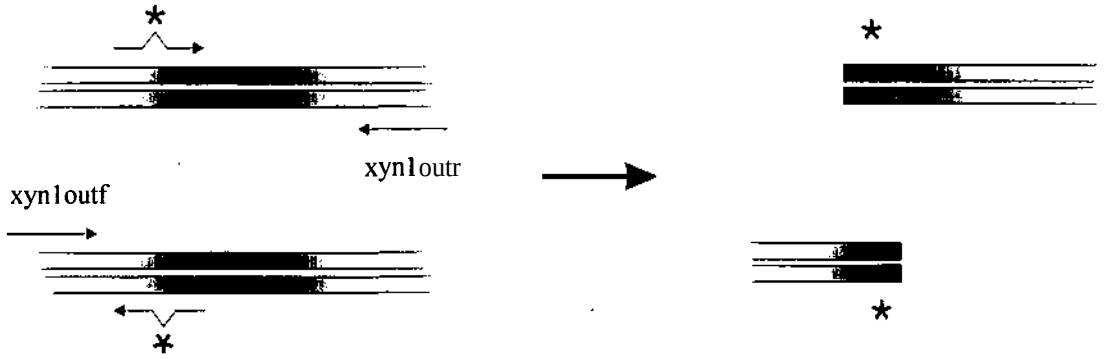
Introduction of specific mutations into cis *acting elements of the* xyn1 *promoter*

For the introduction of the mutations into the *xynl* promoter a two-step *in vitro* site directed mutagenesis strategy using PCR was chosen. In the first step, a primer harbouring the desired alterations in the motif was used together with an outer primer to amplify one part of the promoter, including the motif itself. In a separate second PCR reaction a primer covering the opposite strand and harbouring the same mutation was used together with the opposite outer primer to amplify the second part of the promoter. Both fragments were purified by agarose gel electrophoresis (2% agarose in 1x TAE) the bands excised and eluted using the QIAEX gel extraction kit provided by QIAGEN.

These fragments, which were overlapping at the mutated site, were then combined in a second step in another PCR reaction together with both outer primers (xyn1outf, xyn1outr) to yield the whole *xynl* promoter, now bearing the desired mutation. The outer primers were designed containing a unique restriction site each *(Sal* I in the 5' primer xyn1outf, *Xba I* in the 3' primer xynloutr), so that directional cloning of the resulting fragment would be possible.

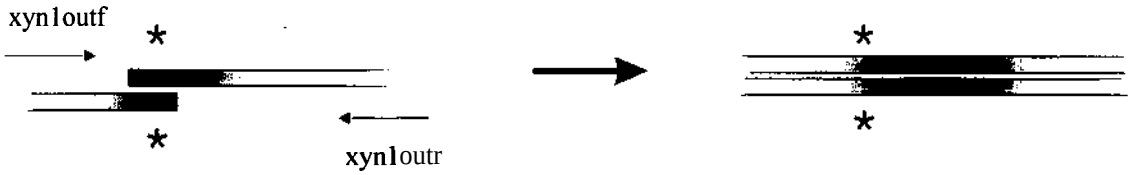
In vitro Mutagenesis by PCR

Step 1:



In the first step the promoter was amplified by PCR in two separate reactions using one outer primer and a primer bearing the desired mutation (marked with an asterisk), yielding two fragments overlapping at the mutated site.

Step 2:



In the second step the two fragments were combined and completed in a PCR reaction using now both outer primers, yielding the full-length mutated promoter fragment.

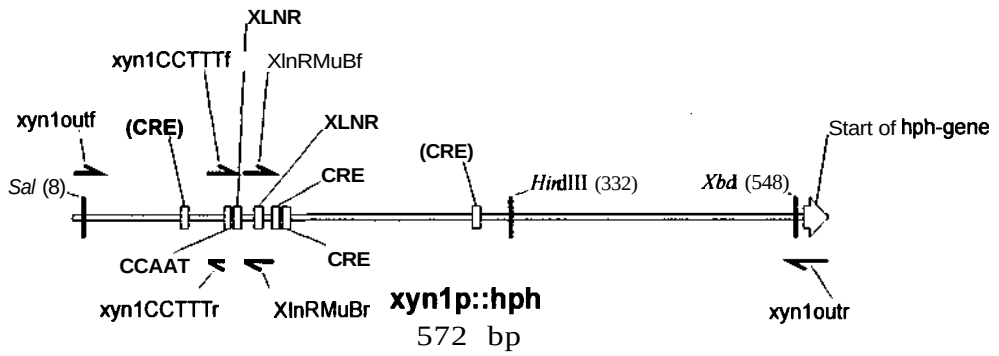


Fig. 5: The upper panel shows the principle of the site directed mutagenesis strategy used. The lower panel gives an overview of the primer positions and the binding sites in the ***xyn1*** promoter fragment amplified from the pRAMB vectors. The ***hph*** sequence lies behind the *Xba* I site and is removed upon *Sal* I / *Xba* I digest.

Primers for mutagenesis:

Outer primers

xyn1outf

5' - GTC GAG GTC GAC GCA AAT GG - 3'

(*Sal* I restriction site underlined)

xyn1outr

5' - GTG AGT TCA GGC TTT TTC ATC TAG AGA TG - 3'

(*Xba*I restriction site underlined)

Mutational primers

Mutation ofthe XlnR like binding motif (XLNR-MUA,XLNR-MUB):

xlnRMuA-fwd

5' – GAT TGG CAG TCT AAA TGC GAC ATC TT - 3'

xlnRMuA-rev

5' - AAG ATG TCG CAT TTA GAC TGC CAA TC – 3'

This primer set changes the 5' part of the XlnR double site from the consensus sequence GGCTAA to GTCTAA, what clearly weakens protein/DNA complex formation on the whole motif in EMSA experiments (Wacenovsky 1998).

XlnRMuBf

5' - GCG ACA TCT TAG ACG GAT GCA C - 3'

XlnRMuBr

5' - GTG CAT CCG TCT AAG ATG TCG C - 3'

This primer set changes the 3' part of the XlnR double site from the consensus sequence GGCTAA to GTCTAA, causing a G to T transversion at the second G of the consensus,

which has been reported to be essential for XlnR binding in *A. niger* (van Peij, Visser *et al.* 1998), and what results in complete loss of protein/DNA complex formation on the whole motif in EMSA experiments (Wacenovsky 1998).

Mutation of the CCAAT box (CCTTT):

xyn1CCTTTf
5'– GCC CCT TGA CTT GAA AGG CAG – 3'

xyn1CCTTTr
5' - CTG CCT TTC AAG TCA AGG GGC - 3'

xyn1CCTTTf-2
5'– GCC CCT TGA CTT GAA AGG CAG GCT – 3'

xyn1CCTTTr-2
5' - AGC CTG CCT TTC AAG TCA AGG GGC - 3'

This primer sets change the CCAAT box to CCTTT what abolishes protein/DNA complex formation on this site completely in EMSA experiments (Zeilinger, Mach *et al.* 1996).

In the case of the CCAAT to CCTTT mutation a second primer pair had to be constructed (xyn1CCTTT-2), because the one chosen first always caused the PCR product to carry an additional adenin directly 3' to the primer site, apparently resulting from the terminal transferase activity of the *Taq* polymerase. The problem was solved letting the new primer pair end before a thymin in the template sequence.

The template used for single mutations was pRAMB1, a vector consisting of a pUC19 backbone with an insert of the complete *xyn1* promoter in front of the *E. coli hph* structural gene. For the double mutations including the altered Cre1 site, pRAMBl 1, which is the same as pRAMB1, the only difference being a deletion of four bases in the double Crel site of the *xyn1* promoter (Mach, Strauß *et al.* 1996), was used. For the other double mutations and the triple mutation the respective intermediate constructs (the pXIP vectors carrying the already mutated *xyn1* promoter in a pGEM-T backbone as described below) were taken as template.

Mutation	Template
WT	—
XLNR-MUA	pRAMBl
XLNR-MUB	pRAMBl
CCTTT	pRAMBl
CRE	—
XLNR-MUB + CCTTT	pXIP-1
XLNR-MUB + CRE	pRAMB11
CCTTT + CRE	pRAMB11
XLNR-MUB + CCTTT + CRE	pXIP-3

Table 2: Overview of the template vectors used in the site directed mutagenesis PCR.

PCR reactions and cycling conditions:

Stage 1:

Reaction mixtures

36,5 µl	SB
5 µl	10× Herculase buffer (Stratagene)
5 µl	2 mM dNTPs
1 µl	pRAMBl / pRAMBl 1 / pXIP-1 / pXIP-3 template DNA
1 µl	xyn1outf primer (10 µM)
1 µl	xlnRMuBr primer (10 µM) or xyn1CCTTTr-2 primer (10 µM) or xlnRMuA-rev primer (10 µM)
0,5 µl	Herculase (5 u/µl; Stratagene)
50 µl	total

36,5 µl	SB
5 µl	10× Herculase buffer (Stratagene)
5 µl	2 mM dNTPs
1 µl	pRAMBl / pRAMB11 / pXIP-1 / pXIP-3 template DNA
1 µl	xlnRMuBf primer (10 µM) or xyn1CCTTTf-2 primer (10 HM) or xlnRMuA-fwd primer (10 HM)
1 µl	xyn1outr primer (10 µM)
0,5 µl	Herculase (5 u/µl; Stratagene)
50 µl	total

PCR programm

Temperature	Time	Cycles
92°C	2 min	1
92°C	30 sec	30
60°C	30 sec	
72°C	1 min	
72°C	10 min	1
4°C	oo	–

The two fragments resulting from this first stage PCR were purified by agarose gel electrophoresis (2% agarose, 1 x TAE), excised from the gel and eluted using a QIAEX gel extraction kit (QIAGEN). The fragments were finally resuspended in 20 ul SB.

Stage 2:

Reaction mixture

66 µl	SB
10 µl	10× *Taq* buffer (Stratagene)
10 ul	2 mM dNTPs (Promega)
5 µl	fragment 1
5 µl	fragment2
2 µl	xyn1outf primer (10 µM)
2 µl	xyn1outr primer (10µM)
0,5 ul	*Taq*2000 polymerase (5 u/µl; Stratagene)
100,5 µl	total

PCR programm

Temperature	Time	Cycles
95°C	1 min	1
95°C	1 min	12
60°C	1 min	
74°C	1 min	
74°C	7 min	1
4°C	oo	–

The 572 bp *xynI* promoter fragments resulting from this second stage were purified using agarose gel electrophoresis as well, the bands excised and eluted using the same QIAEX gel extraction kit as in the first stage and resuspended in 20 ul SB.

4 ul of the PCR product were then ligated together with 1 ul pGEM-T (50 ng/µl ; Promega) using 5 ul TaKaRa ligation kit version 2 solution I, yielding a total reaction volume of 10 µl. The mixture was incubated at 17°C for about an hour and afterwards transformed into chemically competent *E. coli* JM109.

The intermediate constructs resulting from this procedure were named pXIP.

Building the **xyn1$_p$::goxA** *reporter gene constructs*

The **pXIP plasmids** were digested with *Sal* I / *Xba* I yielding a 540 bp *xynl* promoter fragment bearing the desired mutations.

2,5 ul of this fragment was ligated with 2,5 ul of a *Xba* I / *Xho* I fragment of the vector pSJ3 bearing the *goxA* structural gene in a **pUC19** backbone using again 5 **ul** TaKaRa ligation kit version 2 solution I for the reaction. The ligation was performed at **17°C** for about two hours. The whole ligation reaction (10 ul) was transformed into chemically competent *E. coli* JM109.

The resulting final constructs were named **pGXI,** carrying the mutated *xynl* promoter in front of the *goxA* structural gene.

Plasmid	Mutation
pGXI-WT	—
pGXI-1	XLNR-MUB
pGXI-2	CCTTT
pGXI-3	CCTTT + CRE
pGXI-4	XLNR-MUB + CRE
pGXI-5	XLNR-MUB + CCTTT
pGXI-6	XLNR-MUB + CCTTT + CRE
pGXI-8	CRE
pGXI-9	XLNR-MUA

Table 3: The mutations in the *xyn1* promoter present in the different reporter gene vectors.

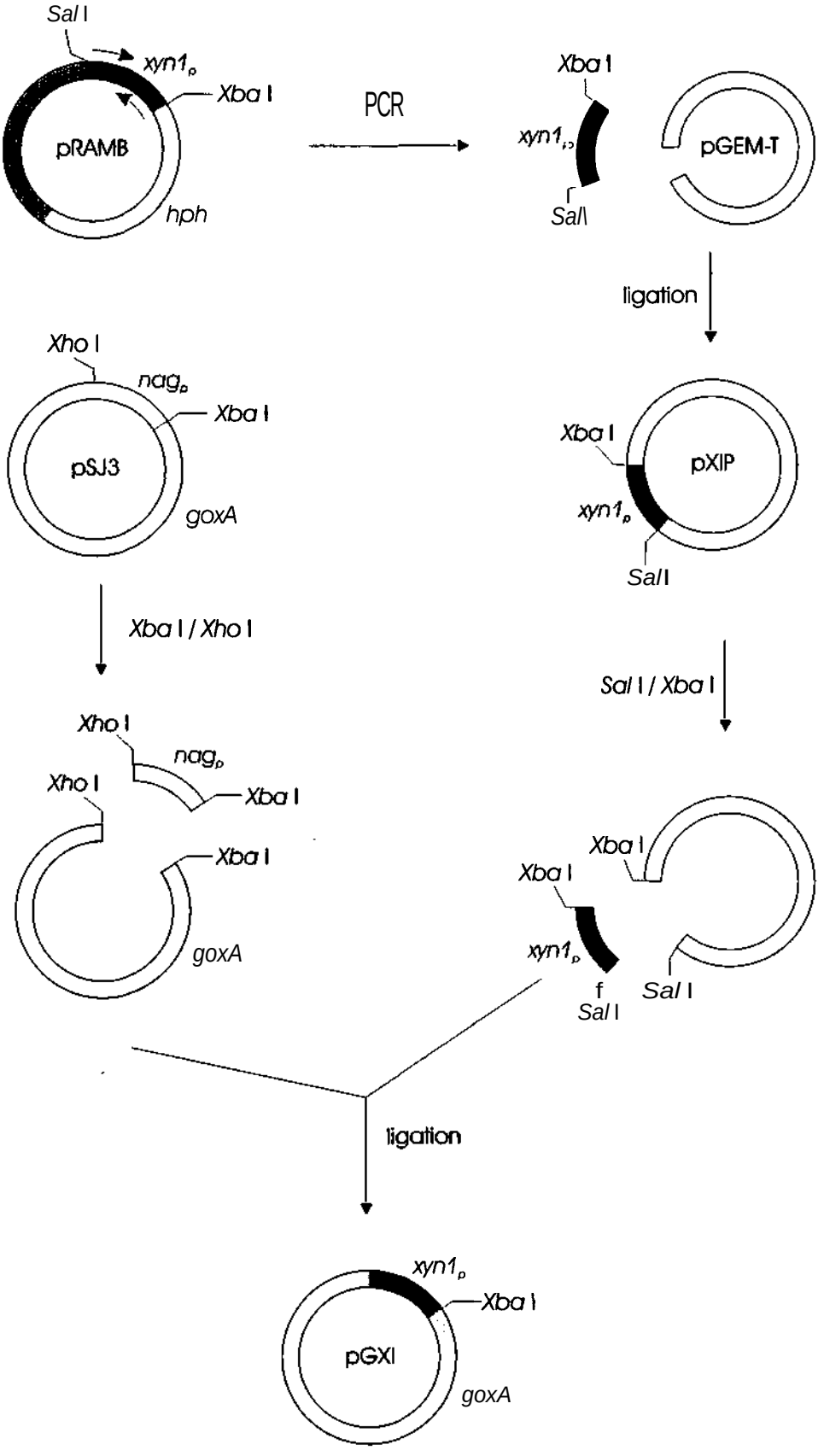


Fig. 6: Schematic overview of the construction of the reporter gene vectors.

Transformation of T. reesei *with the* xyn1$_p$::goxA *reporter gene constructs*

The pGXI plasmids carrying the *xyn1*$_p$::*goxA* reporter gene constructs were co-transformed together with the pFG1 plasmid (harbouring the *T. reesei pyr4* gene) into the *pyr*$^-$ strain *T. reesei* TU-6 using biolistic particle bombardment on spores plated on selective minimal medium agar. Growing colonies were excised and each of them transferred to a fresh petri dish containing the same minimal medium agar. For sporulation the clones were then grown on malt extract plates, the spores washed down with 0,8% NaCl and streaked out on plates containing minimal medium plus 0,1% peptone and 0,1% TritonX-100. 30-50 single spore colonies from each type of mutation were collected, cultivated on minimal medium agar again and then passaged to mitotic stability growing them at least three times on malt extract agar and minimal medium agar with 0,1% peptone alternating, the percentage of stable strains finally recovered being about 30-50% depending on the mutation introduced.

Consequently the strains emerging from this procedure were called *T. reesei* GXI and subjected to southern screening for heterologous integration of the reporter gene construct. To this end the genomic DNA of 120 different clones was isolated and digested with *Pst* I which has a single restriction site in the transformation plasmid pGXI positioned in the *goxA* structural gene. As probe a 2,4 kb *Hind* III fragment from pGXI-WT was used, covering most of the *xyn1* promoter and the *goxA* structural gene. This allowed to screen for heterologous integration and for copy number at the same time, as the *xyn1*$_p$ part of the probe would hybridise with the original *xyn1* locus proving that it remained intact, and the *goxA* part of the probe would produce two signals for each copy of the reporter gene integrated into the genome.

Clones showing the desired heterologous integration pattern were grown in liquid cultures and checked for GOX activity under inducing (replacement on xylan as sole carbon source) as well as under repressing (direct cultures on glycerol) conditions.

Transformation plasmid	Stable clones	Integration		Single copy	Multi copy	GOX activity
		none	total			
pGXI-WT	17	9	8	5	3	3
pGXI-1	9	2	7	5	2	0
pGXI-2	9	0	9	6	3	3
pGXI-3	12	3	9	5	4	4
pGXI-4	8	1	7	3	4	0
pGXI-5	19	5	14	5	9	4
pGXI-6	21	10	11	4	7	3
pGXI-8	16	4	12	8	4	2
pGXI-9	9	1	8	4	4	3
Total	120 (100%)	35 (29%)	85 (71%)	45 (38%)	40 (33%)	22 (18%)

Table 4: Number and integration type of stable transformants yielded after introduction of the *xyn1*$_p$::*goxA* reporter gene construct by biolistic particle bombardement. All integrations are ectopic.

GOX activities of xyn1 *promoter mutated reporter strains*

Replacement cultures on xylan:

For replacement on xylan 4 g of wet mycelium pregrown for 36 h at 30°C on Mandels-Andreotti medium with 1% (w/v) gycerol as carbon source and 0,1% (w/v) peptone was used, which was washed thoroughly with cold, sterile tap water, transferred into Mandels-Andreotti medium containing 0,5% (w/v) xylan as the only carbon source and incubated further at 30°C on a rotary shaker.

Samples of 1 ml were collected from the cultures, the mycelium separated by centrifugation and the supernatant used for glucose oxidase assay. The glucose oxidase activity was measured colorimetrically by determining the increase of the absorbance at 420 nm, reflecting the increasing concentration of oxidised ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)). The reaction is mediated by horse radish peroxidase,

which degrades the hydrogen peroxide primarily synthesised by the glucose oxidase thereby oxidising the ABTS substrate.

Clone	Mutation	Copy number	Time	Activity [U/ml]
GXI-WT.3	—	1	24 h	26,70
			48 h	27,31
			72 h	31,16
GXI-WT.9	—	1	24 h	7,35
			48 h	8,61
			72 h	9,36
GXI-WT.15	—	1	24 h	11,61
			48 h	12,89
			72 h	29,39
GXI-2.4	CCTTT	1	24 h	23,39
			36 h	23,37
			48 h	24,47
			60 h	24,52
GXI-2.17	CCTTT	1	24 h	7,89
			36 h	8,40
			48 h	8,70
			60 h	8,95
GXI-2.20	CCTTT	1-2	36 h	36,15
			48 h	29,51
GXI-3.21	CCTTT + CRE	2	24 h	19,87
			36 h	20,91
GXI-3.31	CCTTT + CRE	1	24 h	18,13

			48 h	27,25
			72 h	24,15
GXI-5.15	XLNR-MUB + CCTTT	3	24 h	5,72
			48 h	6,17
			72 h	6,64
GXI-6.9	XLNR-MUB + CCTTT + CRE	2	48 h	5,50
			72 h	5,66
GXI-6.12	XLNR-MUB + CCTTT + CRE	1	24 h	7,46
			48 h	8,89
			72 h	9,67
GXI-6.14	XLNR-MUB + CCTTT + CRE	1	24 h	5,76
			48 h	6,10
			72 h	6,27
GXI-8.17	CRE	2	24 h	33,19
			48 h	31,61
			72 h	32,25
GXI-9.3	XLNR-MUA	1	24 h	5,33
			48 h	5,75
			72 h	6,00
GXI-9.9	XLNR-MUA	2	24 h	5,13
			48 h	5,30
GXI-9.11	XLNR-MUA	2 (tandem)	24 h	5,55
			48 h	5,92

Table 5: Glucose oxidase activities in the culture supernatants of the different clones at various time points after replacement on xylan. No activity whatsoever could be detected within any of the **GXI-1** (XLNR-MUB) and the **GXI-4** (XLNR-MUB + CRE) clones. Clone **GXI-2.17** is somewhat out of the row, maybe due to matrix effects of the integration locus.

Direct cultures on glycerol:

Glycerol was chosen as carbon source because it does not induce xylanase formation (and recent findings also point into the direction that it also causes carbon catabolite repression, although not all in the same way as glucose), and in contrast to glucose it is not a substrate for glucose oxidase, therefor not leading to hydrogen peroxide formation in the culture broth during cultivation, which is reportedly toxic to many fungi (e.g. LD_{50} being about 3 mM for *Aspergillus flavus* (Jacks, De Lucca *et al.* 2000)).

Clone	Mutation	Copy number	Time	Activity [U/ml]
GXI-WT.3	—	1	40 h	0,0
			68 h	0,0
GXI-WT.9	—	1	40 h	0,0
			68 h	0,0
GXI-WT.15	—	1	40 h	0,0
			68 h	0,0
GXI-2.4	CCTTT	1	40 h	0,0
			68 h	0,0
GXI-2.17	CCTTT	1	40 h	0,0
			68 h	0,0
GXI-2.20	CCTTT	1-2	36 h	0,0
			48 h	0,0
GXI-3.21	CCTTT + CRE	2	40 h	5,87
			68 h	5,65
GXI-3.31	CCTTT + CRE	1	40 h	5,55
			68 h	5,49
GXI-5.15	XLNR-MUB + CCTTT	3	40 h	0,0
			68 h	0,0

GXI-6.9	XLNR-MUB + CCTTT + CRE	2	40 h	0,0
			68 h	0,0
GXI-6.12	XLNR-MUB + CCTTT + CRE	1	40 h	0,0
			68 h	0,0
GXI-6.14	XLNR-MUB + CCTTT + CRE	1	40 h	0,0
			68 h	0,0
GXI-8.17	CRE	2	40 h	10,98
			68 h	12,05
GXI-8.26	CRE		40 h	5,17
			68 h	5,18
GXI-9.3	XLNR-MUA	1	40 h	0,0
			68 h	0,0
GXI-9.9	XLNR-MUA	2	40 h	0,0
			68 h	0,0
GXI-9.11	XLNR-MUA	2 (tandem)	40 h	0,0
			68 h	0,0

Table 6: Glucose oxidase activities in the culture supernatants of the different clones at various time points upon growth on glycerol. None of the **GXI-1** (XLNR-MUB) and the **GXI-4** (XLNR-MUB + CRE) clones tested showed any detectable activity on glycerol as well. In strain GXI-8.17 both copies of the reporter gene construct seem to be active, hence exhibiting about the twofold basal activity compared to other clones.

Cloning of the *T. reesei* gene (*xyr1*)homolog to *xlnR* of *A. niger*

As the XlnR like site in the *xyn1* promoter did not only prove functional in the way that it can bind the XlnR protein form *A. niger* and that complexes from cell-free extracts are formed specifically on this site, but as well essential for *in vivo* gene expression driven by this promoter, the next step was to identify and clone the corresponding *trans* acting factor.

Because EMSA experiments showed that XlnR from *A. niger* could bind to the double GGCTAA motif in the *xynl* promoter (Wacenovsky 1998), it seemed likely that the *T. reesei* factor occupying this site could show significant similarity to XlnR, at least in the DNA binding domain. So the first approach was to use a 485 bp *BamH* I / *Xho* I cDNA fragment of the *A. niger xlnR* gene (spanning the zinc cluster domain and the adjacent coiled coil domain of the protein) as a probe for screening a *T. reesei* QM9414 genomic phage library.

Heterologous screening of a T. reesei *genomic library*

First of all, to investigate if a heterologous screening could be performed at all, genomic DNA was prepared from *T. reesei* QM9414, digested with *Eco*R I, *Sal I* and *BamH* I, separated on an agarose gel, blotted to a HYBOND N+ membrane and hybridised over night at 58°C with a 485 bp *BamH* I / *Xho* I fragment of the *A. niger xlnR* structural gene excised from pCOW-1, radio labelled by random priming. As specific signals could be detected in this southern blot, it was decided to proceed to the heterologous screening.

An apt dilution of the *T. reesei* genomic phage library was used to infect *E. coli* strain ER1647. After an hour of incubation at 37°C the bacteria were added to 3 ml top agarose and plated out on LB agarose bottom. After the cultures were incubated for about 7 hours at 37°C they were kept over night at +4°C. The next day the plaques were transferred to a HYBOND N+ membrane and the DNA denaturated in 1,5 M NaCl / 0,5 M NaOH and fixed to the membrane via UV crosslinking. The blots were hybridised with the same radio labelled 485 bp fragment of the *A. niger xlnR* structural gene as used in the preceding experiment under the same conditions (hybridisation over night at 58°C). Washing was done two times for thirty minutes using 2x SSC / 0,1% SDS and two times for thirty minutes using 1 x SSC / 0,1% SDS at 58°C. 16 plaques giving a clear signal on the x-ray film were picked and suspended in 500 ul SM buffer + 20 ul $CHCl_3$. A second screening round was performed infecting 100 ul *E.*

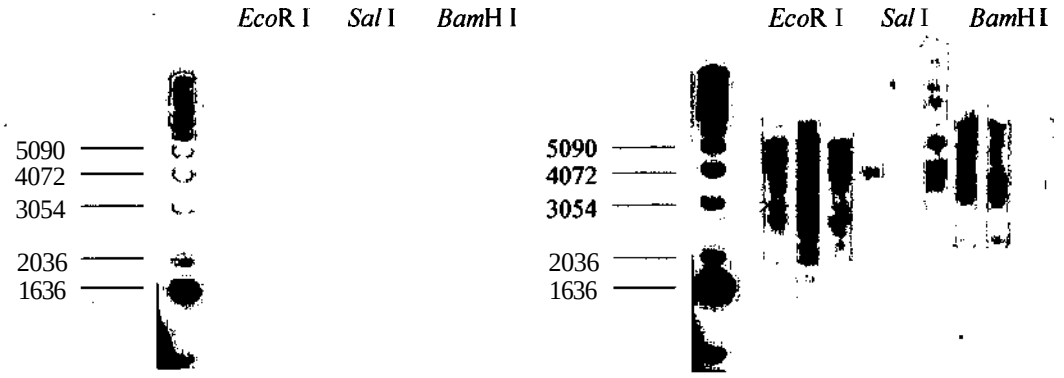
coli ER1647 with 100 ul phage dilution of 3×10^{-3}, 6×10^{-3} and 6×10^{-4} respectively, intended to yield about 100 plaques per plate. Plaques from this secondary screening were used to infect *E. coli* BM25.8, a strain converting phage library DNA to a plasmid. The bacteria were plated out on LB / Amp agar and incubated over night at 37°C. Resulting colonies were grown in 3 ml LB / Amp, the plasmids isolated and transformed into *E. coli* JM109 for amplification. The amplified plasmids were digested using selected restriction enzymes, the resulting DNA fragments separated on an agarose gel, blotted on a HYBOND N+ membrane and southern hybridised in the same way as it was done in the library screening.

Fragments showing hybridisation with the probe were eluted from the gel using a QIAGEN gel extraction kit and ligated into the multiple cloning site of pUC19. The plasmids emerging from the ligation were amplified, checked for restriction pattern and sequenced.

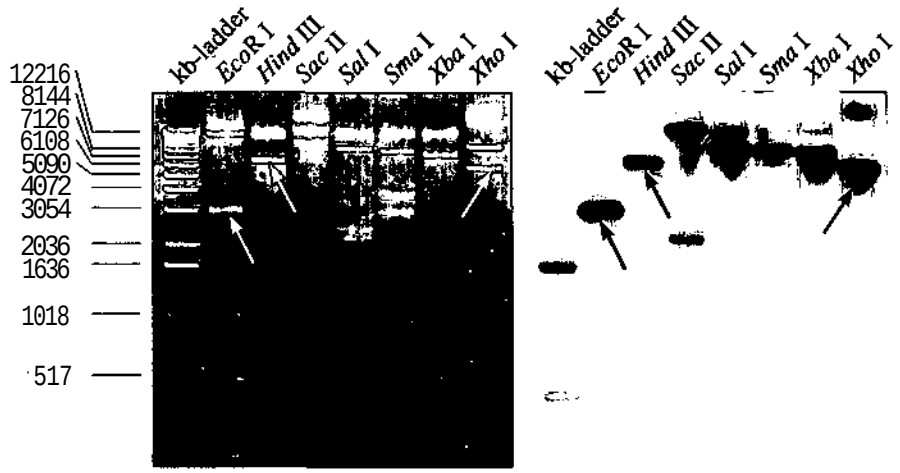
Unfortunately not one single plasmid isolated carried an insert showing any similarity to the *A. niger xlnR* gene. In some cases bacterial sequences from *E. coli* or sequences bearing no similarity whatsoever were found, in other cases the insert has been lost completely.

So it was tried to use different plasmids for cloning (e.g. pBLUESCRIPT) or a different bacterial host for transformation *(E. coli* DH5α). To avoid recombinational events even an *E. coli* strain was tried, which had no recombinases left at all *(E. coli* SURE2, STRATAGENE). In this case, transformation did not yield a single colony in multiple experiments (although a transformation using a control plasmid showed good results). Due to this, the strategy of heterologous screening was finally abandoned.

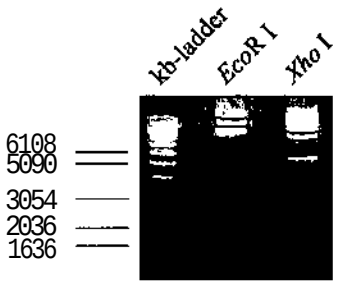
Also RT PCR with primers deduced from the *A. niger xlnR* zinc cluster sequence (as it has been shown to be widely conserved among many fungal species) and mRNA isolated from *T. reesei* cultures grown on xylose, PCR screening of a cDNA phage library (HYBRIZAP®-2.1) produced from *T. reesei* QM9414 xylose cultures specifically for this purpose (and with respect to a possible future approach employing the yeast two hybrid system) with various sets of degenerated and not degenerated primers designed according to sequence similarities between the *A. niger xlnR* gene and an EST (expressed sequence tag) found in the genome of *Metarhizium anisopliae* and a wide range of cycling conditions as well as efforts with linker mediated PCR did not succeed. Another approach was started when the *Neurospora crassa* genome became publically available (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/).



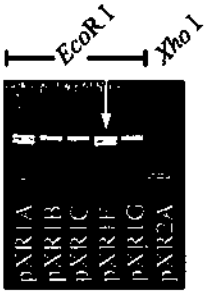
Southern blot of *T. reesei* genomic DNA digested with the enzymes indicated above and hybridized with a 480 bp fragment of the *xlnR* gene from *A. niger*.
Left. Exposure about 3 days. *Right* Exposure about 8 days.



Left. Digestion of the plasmid resulting from the screening of the *T. reesei* genomic library with various enzymes. Arrows are indicating bands which hybridize with high specifity with the *xlnR* probe.
Right. Heterologous southern hybridization of the digestion fragments with the *xlnR* probe.



Digestion of the same plasmid after amplification for QIAEX elution of specific band.



Digestion of plasmids obtained from different clones of £. *coli* JM109 after transformation with excised fragments (one 3 kb and one 5-6 kb) cloned into pUC19 (yielding pXR1 and pXR2 respectively).
The plasmid indicated by an arrow (pXR1F) was sequenced.

Fig. 7: The unsuccessful attempt to clone the *T. reesei* factor homolog to XlnR of *A. niger* by heterologous screening of a *T. reesei* genomic library. No sequence bearing any similarity to the *xlnR* gene could be recovered from any of the numberous clones analysed.

PCR screening of T. reesei *genomic DNA*

By searching the *Neurospora crassa* genomic database with the BLAST programm (http://www.ncbi.nlm.nih.gov/BLAST/) for homologs of the *A. niger xlnR* gene, a contig with an ORF with stretches of high similarity to this gene was found. This putative protein contained a Cys6 zinc cluster of the GAL4 type near the N-terminus that shared striking similarity with that of the *A. niger* factor (92% identity), and also the C-terminus showed a highly conserved region (86% identity). The overall similarity was 47,6% identical amino acids (58,0% positives).

Based on the sequence comparison between the two proteins from *A. niger* and *N. crassa* degenerated primers were designed to screen the *T. reesei* genome for a corresponding regulatory factor using PCR.

Primers for screening:

xlnR-ncr3-fwd (DDVVTYIH)
5' - GA(C/T) GA(C/T) GTI GTI ACI TA(C/T) AT(A/C/T) CA - 3'

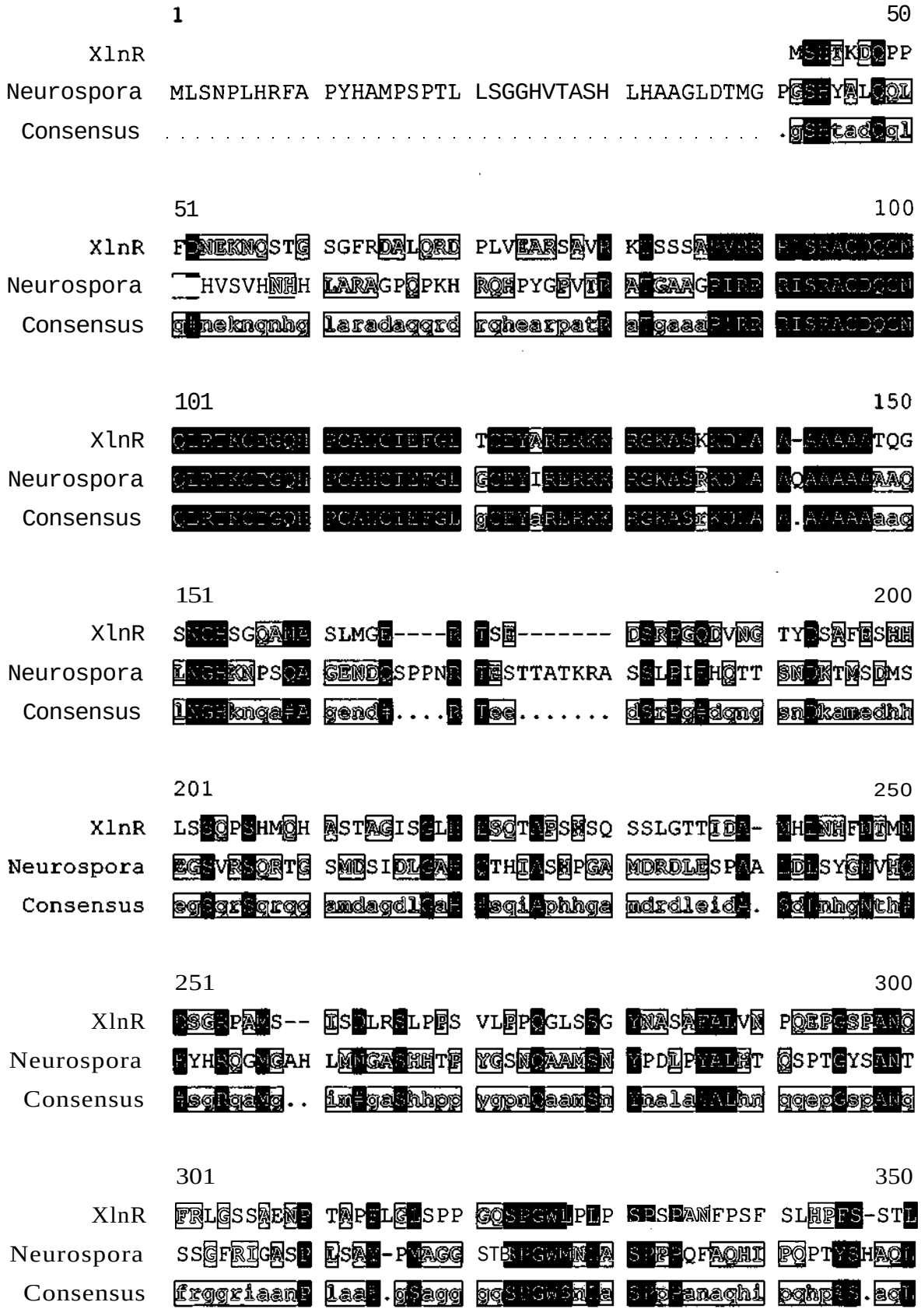
xlnR-ncr4-fwd (TEEEREE)
5' - ACI GA(A/G) GA(A/G) GA(A/G) (A/C)GI GA(A/G) GA - 3'

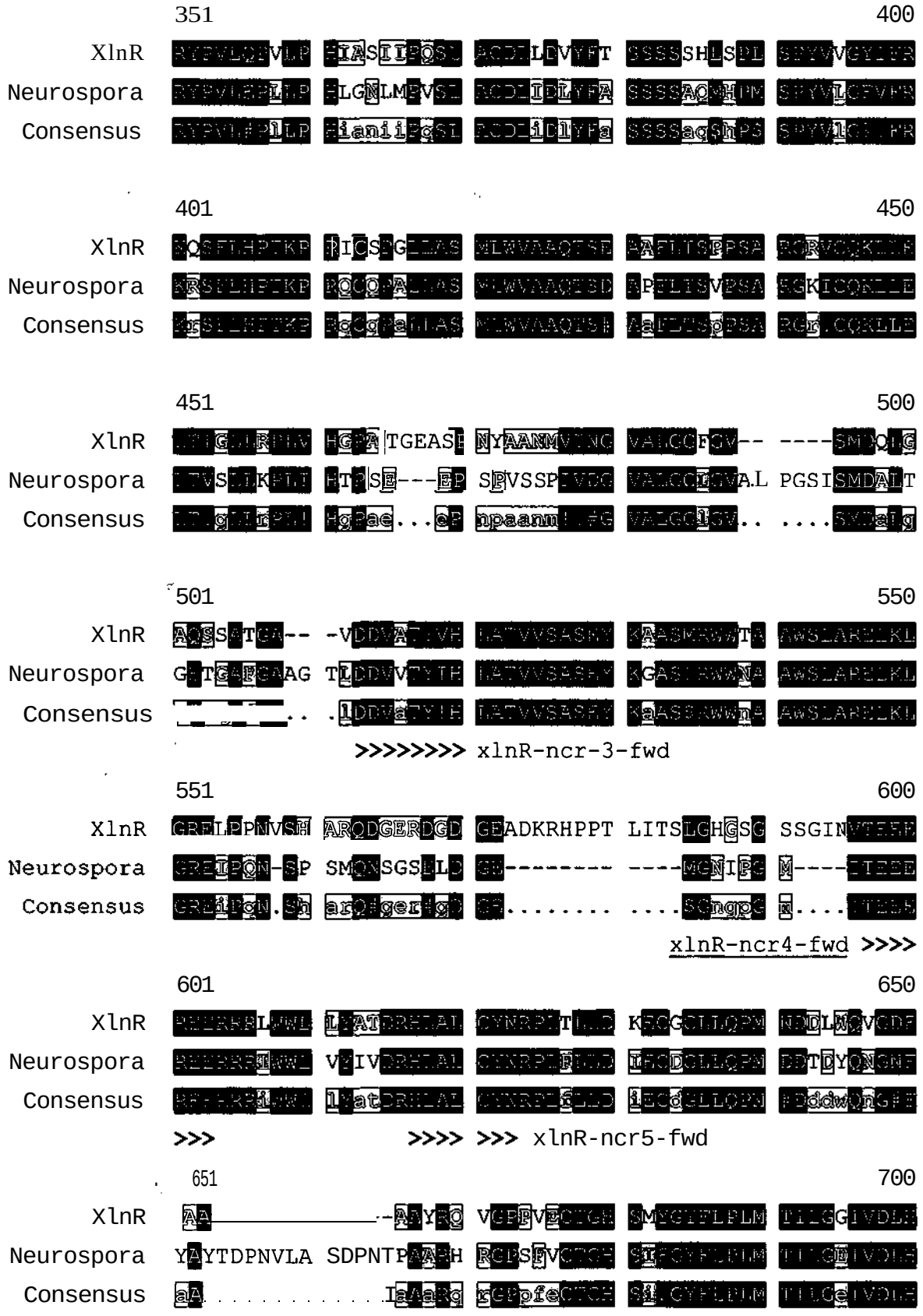
xlnR-ncr5-fwd (HLALCYN)
5' - CA(C/T) (C/T)TI GCI (C/T)TI TG(C/T) TA(C/T) AA - 3'

xlnR-ncr6-rev (HEACVVT)
5' - GTI ACI AC(A/G) CAI GC(C/T) TC(A/G) TG - 3'

xlnR-ncr7-rev (FMPFFFGIY)
5' - TA(A/G/T) ATI CC(A/G) AA(A/G) AA(A/G) AAI GGC AT(A/G) AA - 3'

The primers were diluted to 100 pmol/µl (= 100 µM) as a stock solution and used in a final concentration of 15,3 µM.


1 50
XlnR
Neurospora MLSNPLHRFA PYHAMPSPTL LSGGHVTASH LHAAGLDTMG
Consensus
51 100
XlnR
Neurospora
Consensus
101 150
XlnR
Neurospora
Consensus
151 200
XlnR
Neurospora
Consensus
201 250
XlnR
Neurospora
Consensus
251 300
XlnR
Neurospora
Consensus
301 350
XlnR
Neurospora
Consensus


351
400
XlnR
Neurospora
Consensus
401
450
XlnR
Neurospora
Consensus
451
500
XlnR
Neurospora
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501
550
XlnR
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>>>>>>>> xlnR-ncr-3-fwd
551
600
XlnR
Neurospora
Consensus
xlnR-ncr4-fwd >>>>
601
650
XlnR
Neurospora
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>>> >>>> >>> xlnR-ncr5-fwd
651
700
XlnR
Neurospora
Consensus

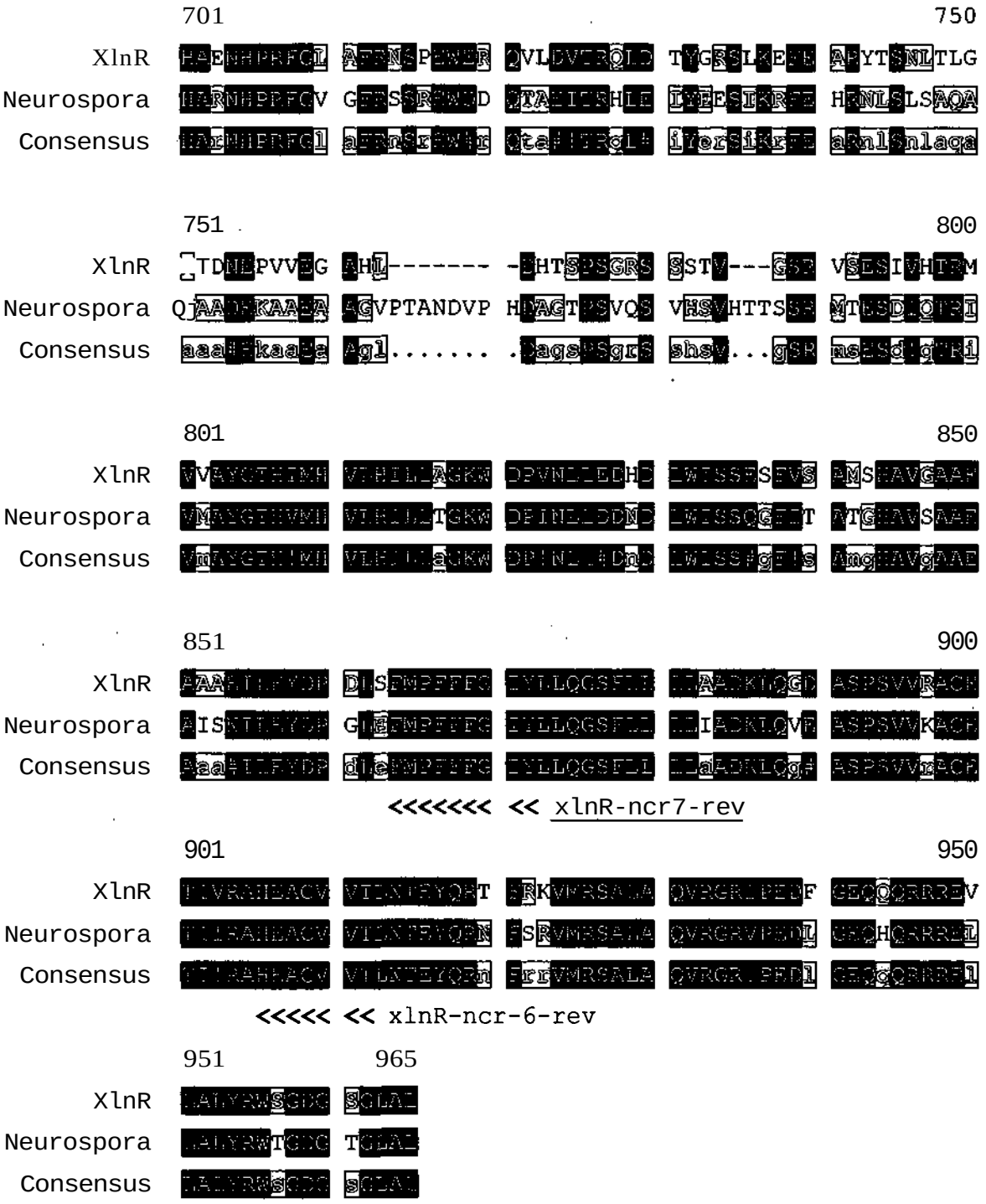

Fig. 8: Alignment of the protein sequence of the *A. niger* XlnR factor with a hypothetical protein derived from the *Neurospora crassa* genomic data base. The positions used for designing screening primers are indicated below the sequence.

The alignment was performed using Multalin version 5.4.1 (multiple sequence alignment with hierarchical clustering; copyright I.N.R.A. France 1989, 1991, 1994, 1996) (Corpet 1988)

Symbol comparison table: blosum62, gap weight: 12, gap length weight: 2

Consensus levels: high=90% low=50%

Consensus symbols: ! is anyone of IV, 1 is anyone of LM, % is anyone of FY, § is anyone of NDQEBZ

Reaction mixture:

24,7 µl	SB
4 µl	10× *Taq* buffer IV (Advanced Biotechnologies)
4 µl	25 mM $MgCl_2$ (Advanced Biotechnologies)
5 µl	2 mM dNTPs (Promega)
1 µl	*T. reesei* QM9414 genomic DNA (1:10)
0,4 µl	forward primer (100 µM)
0,4 µl	reverse primer (100 µM)
0,5 µl	*Taq* polymerase (5 u/µl ; Advanced Biotechnologies)
40 µl	E

PCR programm:

Hot start	95°C	–	–
Denaturation	95°C	1 min	1 cycle
Denaturation	95°C	1 min	30 cycles
Annealing	46-55°C (gradient)	1 min	
Elongation	74°C	1 min	
Completion	74°C	10 min	1 cycle
Hold	4°C	oo	–

Only the combination of primers xlnR-ncr4-fwd and xlnR-ncr7-rev produced a predominant amplimer of approximately 800 bp in length across the whole temperature gradient (which was applied in steps of 2°C), which appeared as the single reaction product at an annealing temperature of about 52°C. A control reaction using this primer combination without template DNA did not yield any amplimer.

This ~800 bp fragment was eluted from an agarose gel by means of the QIAEX gel extraction kit (QIAGEN) and then ligated into the pGEM-T vector using the TaKaRa ligation kit. The resulting plasmid was named pXR50.

Ligation reaction:

4 µl	xlnR-ncr4/7 fragment
1 µl	pGEM-T (50 ng/µl ; Promega)
5 µl	TaKaRa ligation kit version 2 solution I
10 µl	*E*

The whole ligation reaction was transformed into chemically competent *E. coli* JM109, which were then plated out on LB/Amp/IPTG/XGal agar.

Eight positive transformants were selected and grown in liquid culture in 3 ml LB/Amp and the amplified plasmids isolated. A control digest with *BstZ* I (which has a restriction site on each side of the cloning site of the pGEM-T vector) showed that all plasmids carried an insert of the expected length, so four of them were chosen to again transform chemically competent *E. coli* JM109. For each plasmid transformed two clones were grown for a plasmid praparation using the QIAGEN plasmid mini preparation kit.

These eight plamids were subjected to a control digest with *BstZ* I which showed in all cases the presence of the ~800 bp fragment. So two of the plasmids were sequenced (VBC Genomics). They both turned out to be identical in sequence carrying an insert of 812 bp in length that showed a high similarity to a part of the *A. niger* XlnR and the putative *N. crassa* factor.

Therefore this 812 bp fragment was radio labelled by random priming and used as a probe to screen a *T. reesei* QM9414 λBluestar genomic phage library to isolate the full-length gene. Hybridisation and washing was carried out at 64°C, the washing was done using 2× SSC / 0,1% SDS (2x 30 min) and 0,1 x SSC / 0,1% SDS (2× 30 min). The first screening round resulted in 17 positive plaques.

Four of them were picked from different plates, suspended in 1 ml SM buffer and subjected a second screening round where 47 positive plaques could be found.
Eight of these were used to infect *E. coli* BM25.8, a strain designed to convert the DNA carried by the phage into a plasmid. On the whole 49 positive clones then were found on the selective plates, 18 of which were amplified and the respective plasmids isolated.

A digest with *Sac* II, releasing the insert from the vector backbone, showed an identical restriction pattern for all plasmids. The gel was blotted onto a Biodyne B membrane (0,45 µm; PALL) in 10× SSC. Hybridisation of the blot with the radio labelled 812 bp

fragment from the PCR screening at 64°C over night showed specific hybridisation of some of the bands, the hybridisation pattern being all the same for each different plasmid analysed.

Accordingly one of the plasmids was taken, a QIAGEN mini plasmid preparation performed and the insert sequenced as far as of any interest. The sequence gained that way has a length of 4.322 bp and contains an open reading frame (ORF) of 2.932 bp including two putative introns (corresponding to a protein of 934 aa). The deduced protein shows a high similarity to the *A. niger* XlnR factor (47,2% identity, 56,5% positives) as well as to the putative *N. crassa* protein (57,0% identity, 66,7% positives) derived from the genomic data base.


99
198
297
396
495
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693
792
891
990
1089
Met Leu Ser Asn Pro Leu Arg Arg Tyr Ser Ala Tyr Pro Asp Ile Ser Ser Ala Ser Phe Asp Pro Asn Tyr His Gly Ser Gln Ser His
1188
Leu His Ser Ile Asn Val Asn Thr Phe Gly Asn Ser His Pro Tyr Pro Met Gln His Leu Ala Gln His Ala Glu Leu Ser Ser Ser Arg Met Ile Arg
1287
Ala Ser Pro Val Gln Pro Lys Gln Arg Gln Gly Ser Leu Ile Ala Ala Arg Lys Asn Ser Thr Gly Thr Ala Gly Pro Ile Arg Arg Arg Ile Ser Arg
1386
Ala Cys Asp Gln Cys Asn Gln Leu Arg Thr Lys Cys Asp Gly Leu His Pro Cys Ala His Cys Ile
1485
GluPhe Gly Leu Gly Cys Glu Tyr Val Arg Glu Arg Lys Lys Arg Gly Lys Ala Ser Arg Lys Asp
1584
Ile Ala Ala Gln Gln Ala Ala Ala Ala Ala Ala Gln His Ser Gly Gln Val Gln Asp Gly Pro Glu Asp Gln His Arg Lys Leu Ser Arg Gln Gln Ser
1683
Glu Ser Ser Arg Gly Ser Ala Glu Leu Ala Gln Pro Ala His Asp Pro Pro His Gly His Ile Glu Gly Ser Val Ser Ser Phe Ser Asp Asn Gly Leu
1782
Ser Gln His Ala Ala Met Gly Gly Met Asp Gly Leu Glu Asp His His Gly His Val Gly Val Asp Pro Ala Leu Gly Arg Thr Gln Leu Glu Ala Ser
1881
Ser Ala Met Gly Leu Gly Ala Tyr Gly Glu Val His Pro Gly Tyr Glu Ser Pro Gly Met Asn Gly His Val Met Val Pro Pro Ser Tyr Gly Ala Gln
1980
Thr Thr Met Ala Gly Tyr Ser Gly He Ser Tyr Ala Ala Gln Ala Pro Ser Pro Ala Thr Tyr Ser Ser Asp Gly Asn Phe Arg Leu Thr Gly His He
2079
His Asp Tyr Pro Leu Ala Asn Gly Ser Ser Pro Ser Trp Gly Val Ser Leu Ala Ser Pro Ser Leu Arg Tyr His Val Leu Arg Pro Val Leu Leu Asp
2178
Val Arg Asn Ile Tyr Pro Val Ser Leu Ala Cys Asp Gln Met Asp Met Tyr Phe Ser Ser Ser Ser Ser Ala Gln Met Arg Pro Met Ser Pro Tyr Val
2277
Glu Gly Phe Val Phe Arg Lys Arg Ser Phe Leu His Pro Thr Asp Pro Arg Arg Cys Gln Pro Ala Leu Leu Ala Ser Met Leu Trp Val Ala Ala Gln
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Thr Ser Glu Ala Ser Phe Leu Thr Ser Leu Pro Ser Ala Arg Ser Lys Val Cys Gln Lys Leu Leu Glu Leu Thr Val Gly Leu Leu Gln Pro Leu Ile

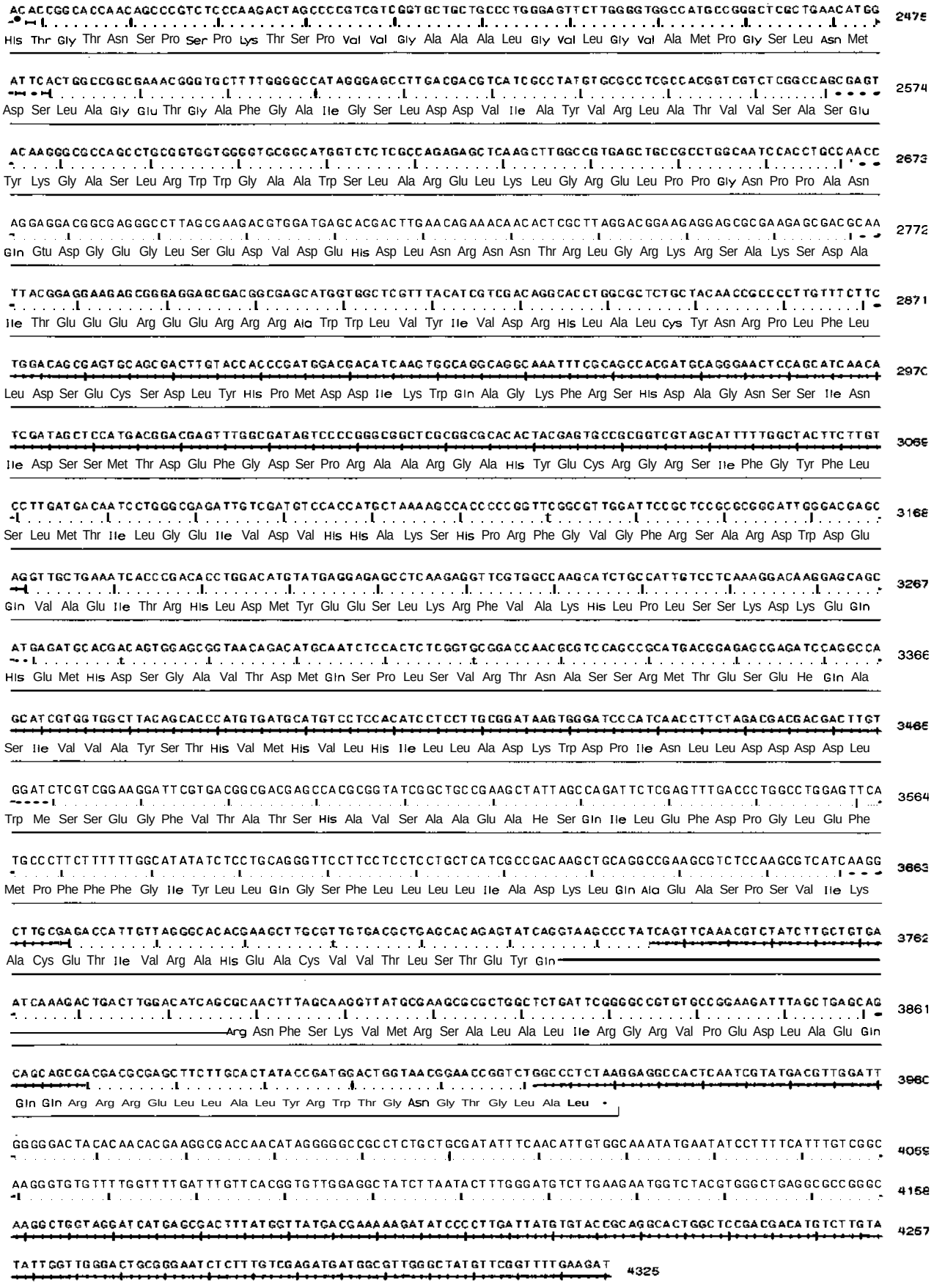


Fig. 9: Genomic sequence of *T. reesei* containing the *xyr1* gene.

Results

Investigation of the function of the cis *acting elements of the* xyn1 *promoter* in vivo

T. reesei strains transformed with the reporter gene construct harbouring the wild-type *xyn1* promoter show strong induction on xylan and no gene expression at all under carbon catabolite repressing conditions, like the *xyn1* gene in the reference strain QM9414.

Mutation of the 5' part of the double xlnR-like binding site (XLNR-MUA)

Introducing a mutation that destroys the upstream part of this motif reduces gene expression driven by the *xyn1* promoter severly to a more or less basal level under inducing conditions.

Like in strains harbouring the wild-type promoter, no gene expression can be observed under carbon catabolite repression.

Mutation of the 3' part of the double XlnR-like binding site (XLNR-MUB)

Rendering the downstream part of this motif void results in a clear loss of expression of the glucose oxidase reporter gene under inducing as well as under carbon catabolite repressing conditions. No GOX activity whatsoever could be detected in the culture supernatants at any time.

Mutation of the CCAAT box (CCTTT)

Converting the CCAAT box in the *xyn1* promoter to CCTTT what abolishes complex formation in EMSA experiments, has no detectable effect on gene expression under inducing conditions. The expression levels found are comparable to that under the control of the wild-type promoter.

Under carbon catabolite repression there is no expression at all, like in the wild-type.

Mutation of the cre1 binding site (CRE)

A non-functional Crel site has no effects under induction on xylan, but relieves the gene controlled by the mutated promoter from carbon catabolite repression leading to a basal level of expression.

Mutation of the 3' part of the double xlnR-like binding site and of the CCAAT box together (XLNR-MUB+ CCTTT)

Althogh the XLNR-MUB mutation abolishes gene expression under all conditions tested, when the CCAAT site is mutated as well, basal expression can be restored under inducing conditions.

The carbon catabolite repression remains intact and so no expression is oberved on respective carbon sources.

Mutation of the 3' part of the double xlnR-like binding site and of the cre1 binding site together (XLNR-MUB + CRE)

When these two motifs are mutated, no gene expression can be detected, neither under inducing, nor under carbon catabolite repressing conditions.

Mutation of the CCAAT box and the crel binding site together (CCTTT + CRE)

The combination of the CCTTT and the CRE mutation exhibits no changes in gene expression under inducing conditions.

On carbon sources normally leading to repression of gene expression strains harbouring these mutations show a basal level of GOX activity.

Mutation of all known functional sites together (XLNR-MUB + CCTTT + CRE)

When all elements known so far to be present in the *xyn1* promoter are knocked out at the same time, basal activity can be observed upon induction by xylan.

Interestingly no activity at all was detected under carbon catabolite repressing conditions

Taken together it can be said, that the XLNR-MUB mutation leads to a complete loss of gene expression under all conditions tested. Only when the CCAAT box is mutated at the same time, basal expression can be restored under inducing conditions. The XLNR-MUA mutation does not abolish gene expression completely, but reduces it strongly.

Mutation of the CCAAT box has no detectable influence on the expression level, unless it is combined with the XLNR-MUB mutation, which - introduced alone - abolishes expression. In this case, basal activity can be restored under inducing conditions, but no gene expression occurs under carbon catabolite repressing conditions, even not if the Cre1 site is

destroyed as well, so that there should be no functional *cis* acting element present any more in the promoter.

Eliminating the function of the Cre1 site generally leads to carbon catabolite derepression, unless the XLNR-MUB mutation is introduced as well. Then again no gene expression can be observed under carbon catabolite repressing conditions.

Mutation	Glycerol	Xylan
WT	—	++
XLNR-MUA	—	+
XLNR-MUB	—	—
CCTTT	—	++
CRE	+	++
XLNR-MUB + CCTTT	—	+
XLNR-MUB + CRE	—	—
CCTTT + CRE	+	++
XLNR-MUB + CCTTT + CRE	—	+

Table 7: Effect of the different mutations oh the glucose oxidase activity level in the respective reporter strains under repressing (glycerol) and inducing (xylan) conditions. Symbols: — no detecable activity; + basal activity; + + elevated level of activity

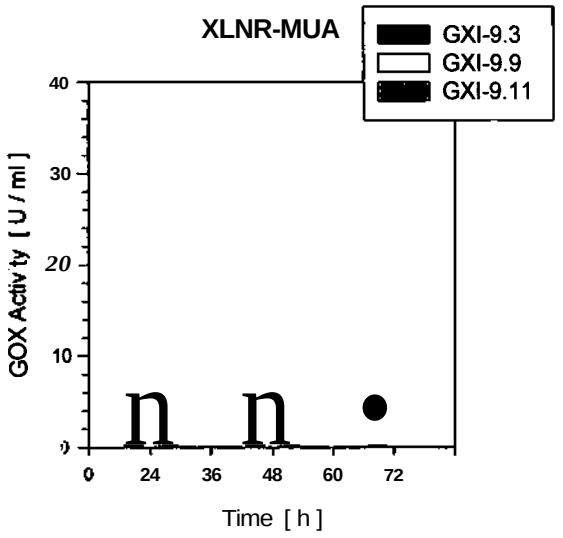


Fig. 10: Comparison of the effect of the mutations introduced into the *xyn1* promoter on the glucose oxidase activities produced by the different reporter strains upon replacement on xylan.

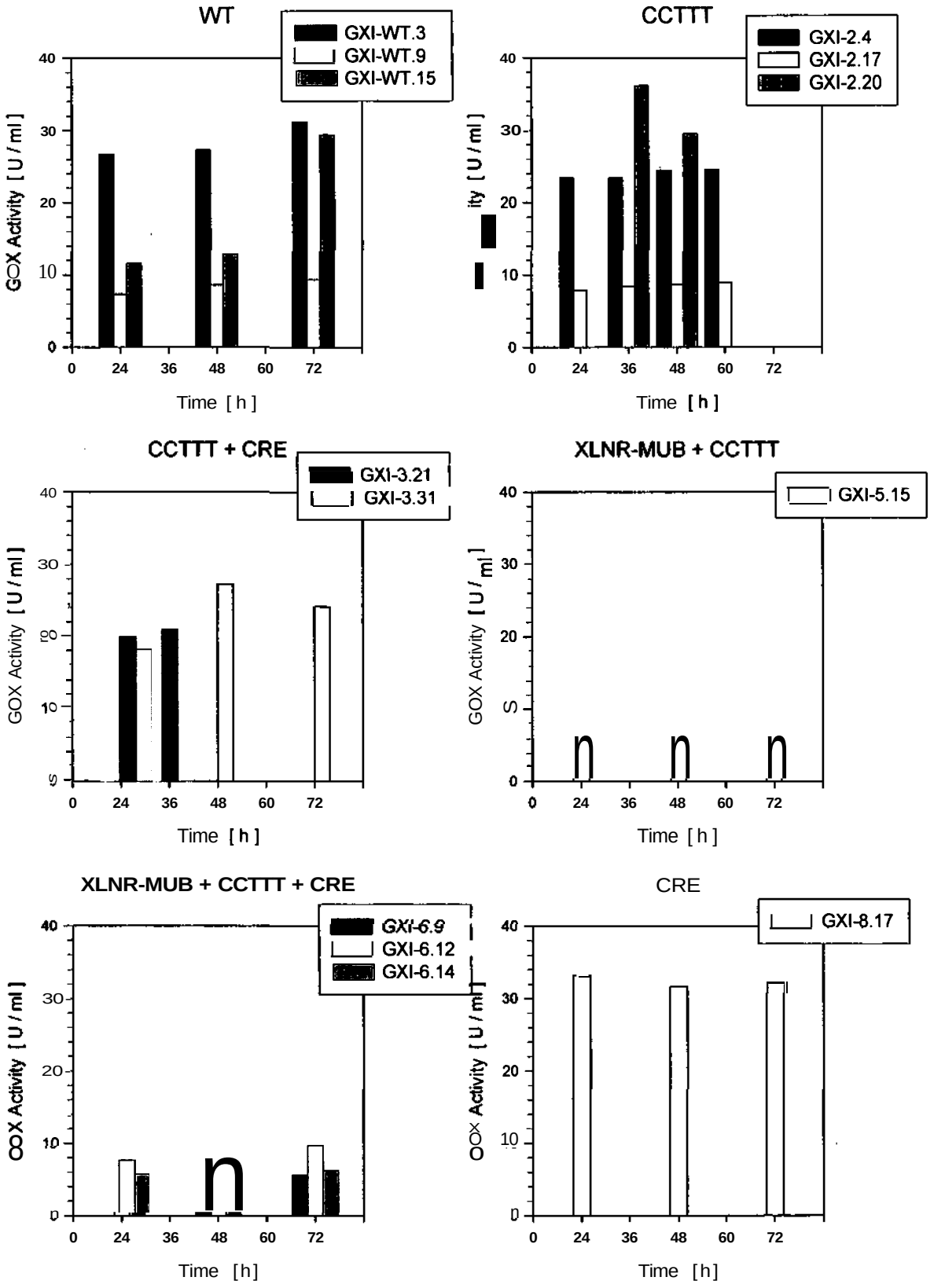


Fig. 10 (*cont.*): Comparison of the effect of the mutations introduced into the *xyn1* promoter on the glucose oxidase activities produced by the different reporter strains upon replacement on **xylan**.

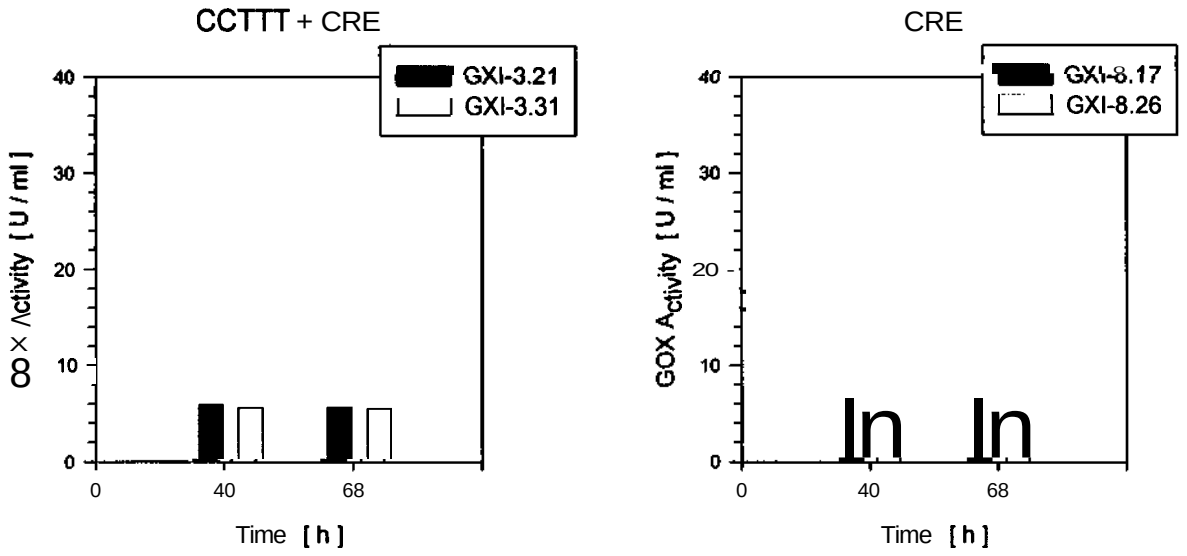


Fig. 11: Comparison of the effect of the mutations introduced into the *xyn1* promoter on the glucose oxidase activities produced by the different reporter strains upon growth on glycerol. Strains not shown here do not exhibit glucose oxidase activity on glycerol.

Cloning of the T. reesei gene *(xyr1) homolog to* xlnR of A. niger

The 4.322 bp sequence resulting from the screening of the *T. reesei* genome consists of an open reading frame (ORF) of 2.926 bp interrupted by two introns and of 998 bp upstream and of 398 bp downstream of the ORF. The gene was named *xyr1* (for xylanase regulator 1), and the coding sequence submitted to the GenBank database. It is available there under the accession number AF479644.

The protein deduced from the ORF is 934 aa long, has a calculated molecular mass of 102,4 kDa and a calculated pI of 6,73. Near to its N-terminus it contains a stretch of amino acids that complies exactly with the consensus sequence Cys-$(Xaa)_2$-Cys-$(Xaa)_6$-Cys-$(Xaa)_5$-Cys-$(Xaa)_2$-Cys-$(Xaa)_6$-Cys of a GAL4 type zinc binuclear cluster DNA binding domain. Also the conserved proline in the second loop reported to be essential for the formation of the cluster in GAL4 (Johnston and Dover 1987) is present at the respective position.

Adjacent to the DNA binding domain on the C-terminal side there is a region with a clustering of the basic amino acids lysine and arginine, which has been considered to take part in nuclear localisation in other cases (Osborne and Silver 1993). The corresponding domain in the *A. niger* XlnR protein has there been proposed to be of a coiled-coil structure (van Peij, Visser *et al.* 1998), but in the *T. reesei* factor structure prediction using the Garnier-Robson algoritm predicts an alpha helical structure for this area, as well as does structure prediction according to the Chou-Fasman algorithm. In spite of this the amino acids postulated to take part in the coiled-coil domain in XlnR are either conserved or replaced with such of similar chemical properties. In the *Saccharomyces cerevisiae* GAL4 and PPR1 factor the region C-terminal to the DNA binding domain has been reported to be involved in homodimerisation of the proteins (Marmorstein, Carey *et al.* 1992; Marmorstein and Harrison 1994).

Interestingly in Xyrl and in the putative protein from *Neurospora crassa* too the region N-terminal to the zinc binuclear cluster domain is exactly 41 aa longer than in XlnR, beginning with six amino acids (MLSNPL) completely conserved between *T. reesei* and *N. crassa* right at the postulated translation start.

Another motif present in the protein is the sequence RRRAWW which is RRRIWW in the putative *N. crassa* factor and resembles closely the RRRLWW motif characteristic for GAL4 family proteins (Suárez, de Queiroz *et al.* 1995) which is found in XlnR. The predicted character of this region is amphipathic according to the algorithm of Eisenberg.

Further down towards the C-terminus there is a region of possible alpha helical structure predicted by the algorithm of Garnier-Robson and Chou-Fasman and amphipathic character according to the algorithm of Eisenberg. This region is thought to participate in protein-protein interaction in XlnR and a leucine (Leu-650) which has been reported to be essential for the functionality of XlnR (van Peij, Visser *et al.* 1998) is present in Xyr1 (Leu-710) and the *N. crassa* hypothetical protein (Leu-708) as well. In the case of XlnR the authors suggest a coiled-coil structure for this area and the hydrophobic amino acids presumed to contribute to this structure are mostly found in Xyrl too.

Two other amino acids found to be absolutely vital for gene activation by XlnR (van Peij, Visser *et al.* 1998) are also conserved among XlnR (Leu-823, Tyr-864), Xyrl (Leu-882, Tyr-923) and the *N. crassa* putative protein (Leu-892, Tyr-933). They are located in the C-terminal region, which is considered to be the trans-activating domain in XlnR. It is spanning the last 160 aa of Xyrl and is nearly identical in all three factors, ending with the sequence GLAL in each.

Sequence comparison between XlnR, the hypothetical protein from *N. crassa* and Xyrl revealed six regions of high similarity. For each of them a search for resembling sequences was performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The databases searched were GenBank, PDB, SwissProt, PIR and PRF.

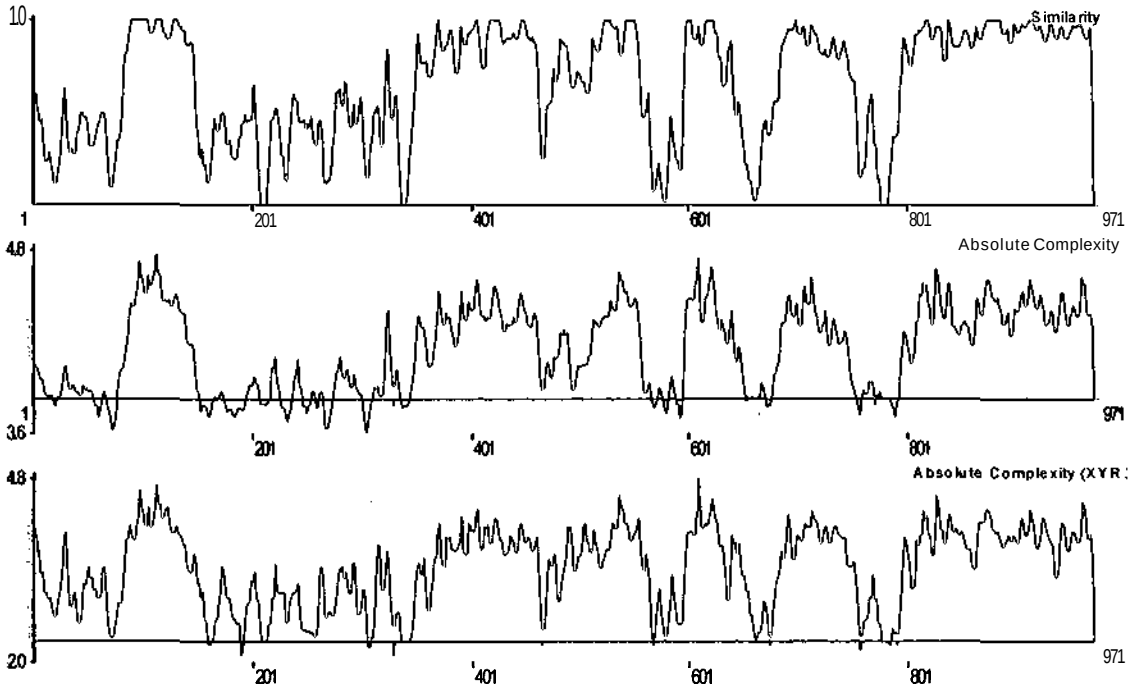


Fig. 12: Graphic display of the similarity between the *Aspergillus niger* XlnR factor, the hypothetical protein from *Neurospora crassa* and Xyr1 from *Trichoderma reesei* (*Hypocrea jecorina*).

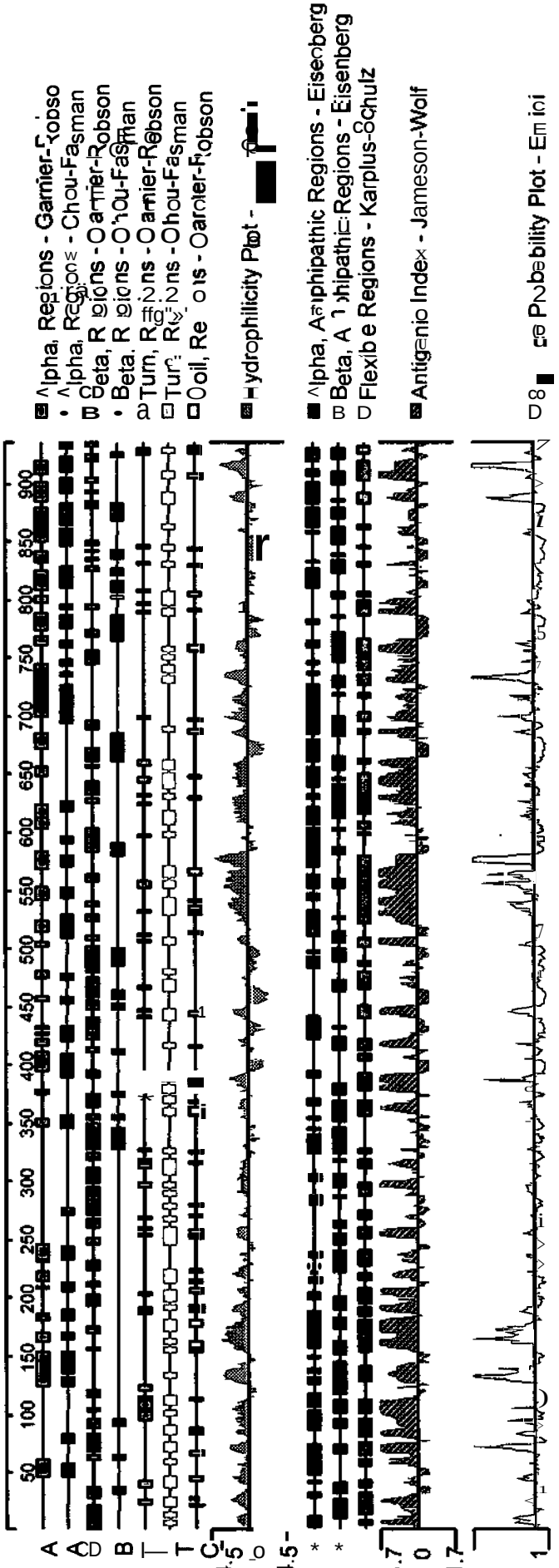


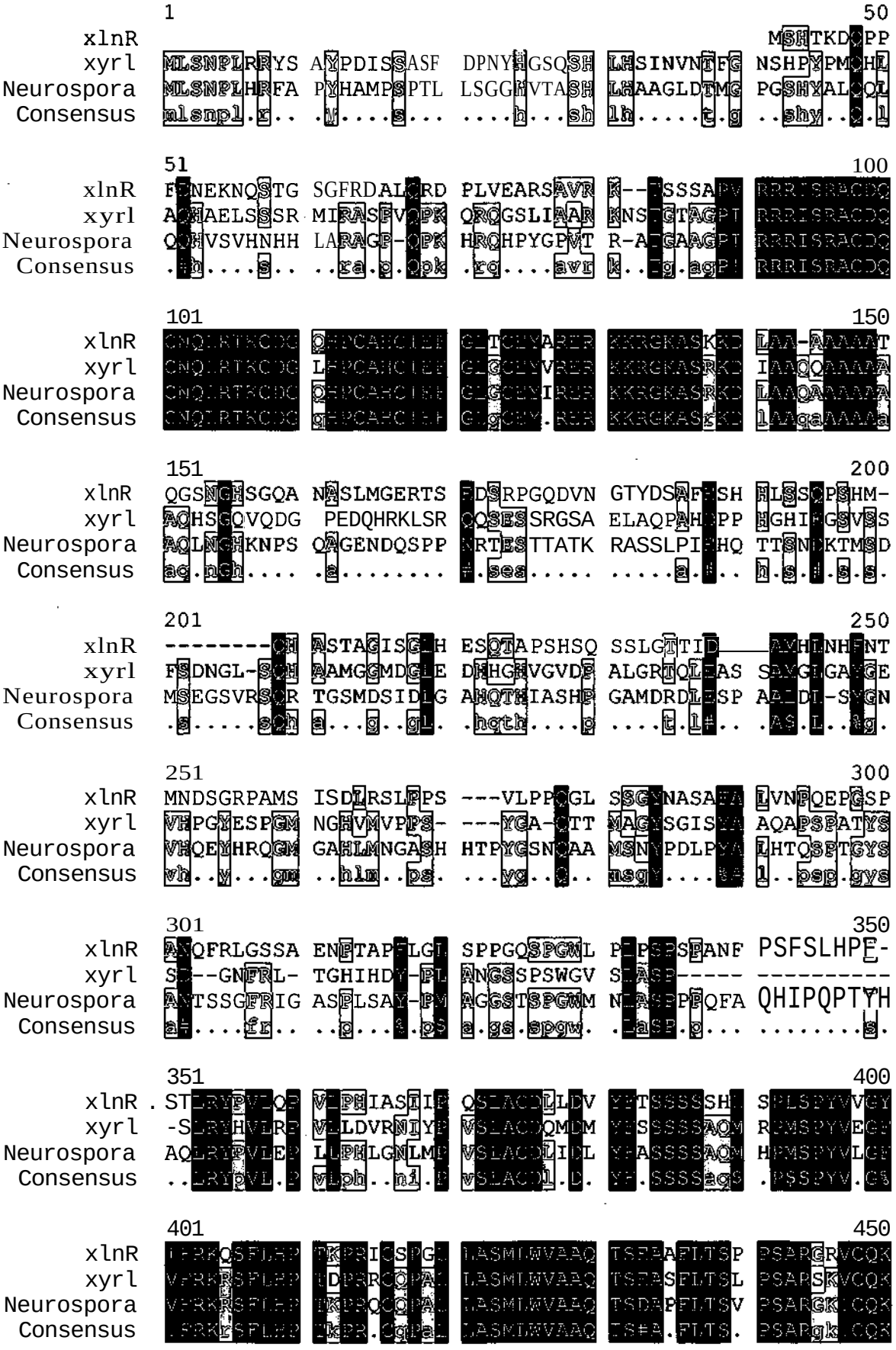
Fig. 13: Predicted secondary structures of Xyr1 derived from the protein sequence according to different algorithms.

The first region showing a striking similarity is the zinc cluster region spanning Arg-91 - Cys-124 and the domain adjacent to its C-terminal end ranging from Glu-125 - Ala-151. The search for this region was done in two steps. First a query comprising Arg-91 - Lys-135 was launched. This produced a result of more than hundred sequences bearing ä Cys_6/Zn_2 binuclear zinc cluster domain, among them the factors XlnR *(A. niger),* Qa-1F, Acu-15 (*Neurospora crassa),* Ginlp, MAL3R, Rdrlp, MAL63, Pdrlp, STB4, Edslp, MSP8 *(Saccharomyces cerevisiae),* Mut3p *(Pichia angusta),* QUTA, FacB *(Emericella nidulans),* Cat8p, Fcrlp *(Candida albicans),* Thilp *(Schizosaccharomyces pombe),* FacB *(Aspergillus oryzae)* and putative factors from *Aspergillus kawachii, A. oryzae, A. nidulans, Podospora anserina, Schizosaccharomyces pombe* and *Neurospora crassa,* all of them having conserved all the cysteines, an alanine before the first cysteine and a proline two positions before the fourth cysteine. Second a separate search for the range Glu-125 - Ala-151 comprising the putative homodimerisation domain was done. This resulted in much less similar sequences. The factors in the databases that produced significant scores were only XlnR *(Aspergillus niger)* and putative proteins from *A. nidulans, A. oryzae* and *A. kawachii.*

All other regions analysed (Val-345 - Thr-439, Asp-487 - Pro-529, Ile-570 - Phe-624, Cys-658 - Val-721 and Ser-757 - Leu-934) likewise showed significant similarities only to XlnR *(A. niger)* and hypothetical transcription factors from *A. nidulans, A. oryzae* and *A. kawachii,* the region Ile-570 - Phe-624 (containing the GAL4 family motif) also giving a hit for a conserved domain specific for fungal transcription factors.

Of course this covers only the factors already submitted to the databases searched. Therefore for example the putative protein derived from the *Neurospora crassa* genomic database which was used for primer design was not among the results, although having an even higher similarity than for instance XlnR.

It is somewhat noteworthy, that aside from protein similarities also the position of the introns (one in the zinc cluster and one in the putative trans-activating domain) is conserved among *xyr1, xlnR* and the putative factor from *N. crassa.* So it should be considered that there also could be the gene for an aspartic acid tRNA located downstream of the *xyrl* gene, as it is the case in *N. crassa,* what would have to be beared in mind a disruption of the *xyrl* gene is desired.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xlnR
xyrl
Neurospora
Consensus
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xlnR
xyrl
Neurospora
Consensus
101 150
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xyrl
Neurospora
Consensus
151 200
xlnR
xyrl
Neurospora
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201 250
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301 350
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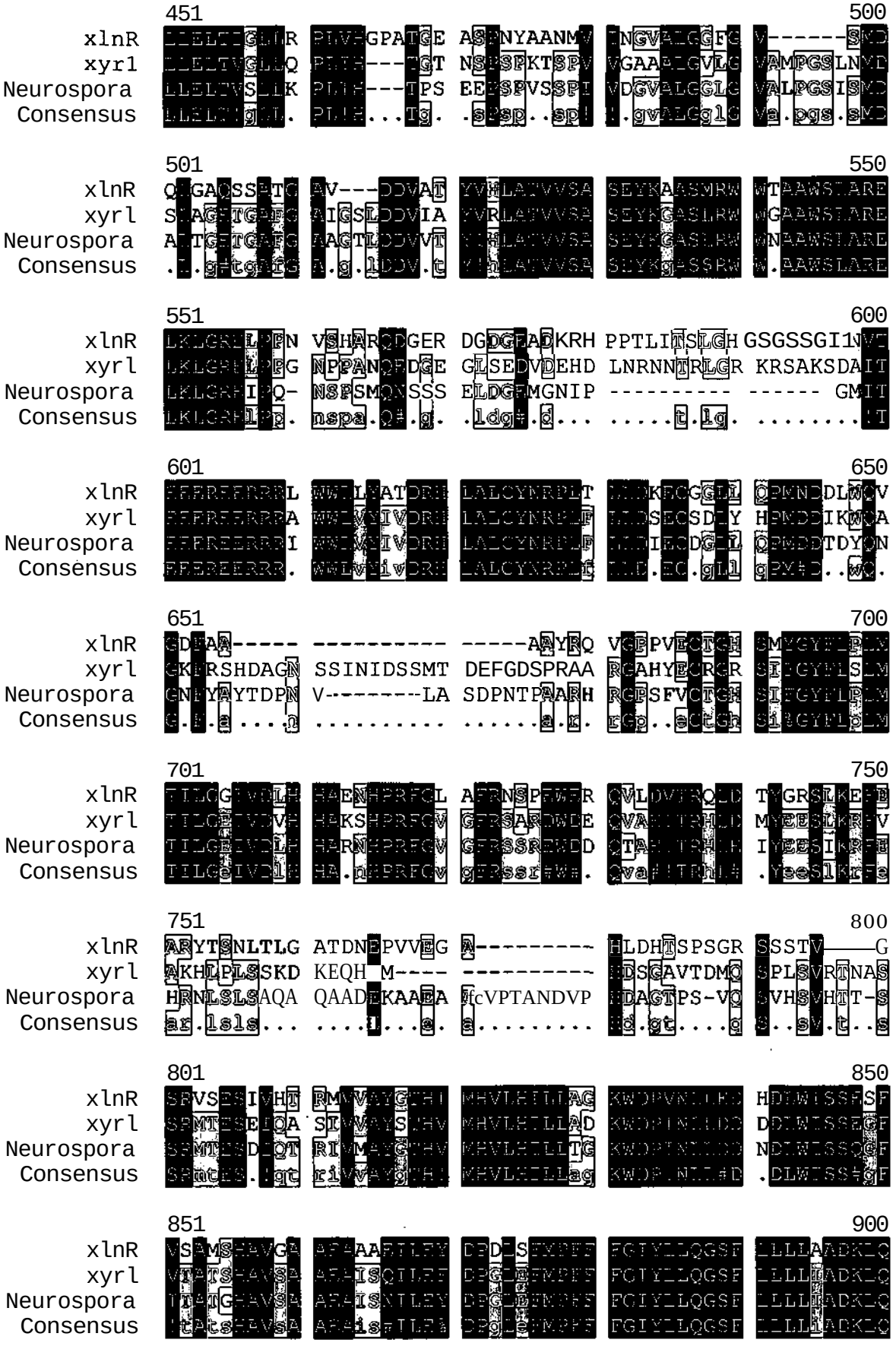
451
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xlnR
xyr1
Neurospora
Consensus
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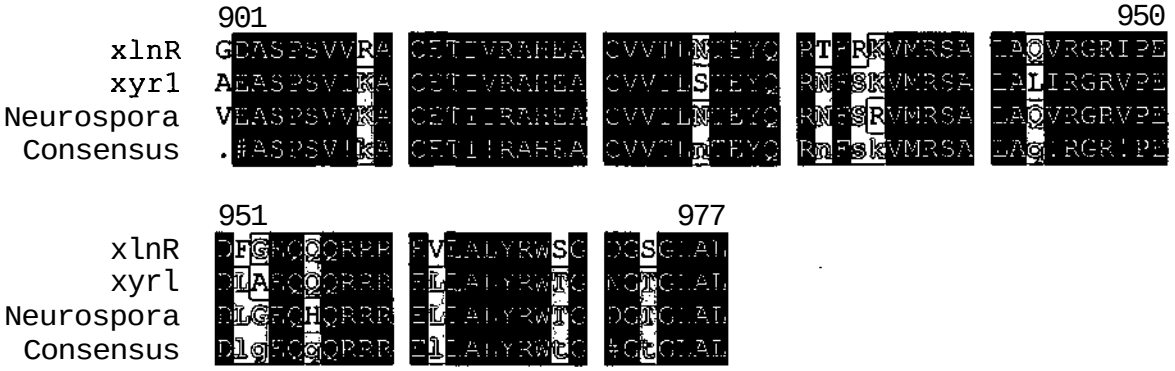

Fig. 14: Sequence alignment of **XlnR, Xyr1** and the hypothetical factor from *Neurospora crassa*.
The alignment was performed using Multalin version 5.4.1 (multiple sequence alignment with hierarchical clustering; copyright I.N.R.A. France 1989, 1991, 1994, 1996) (Corpet 1988)
Symbol comparison table: **blosum62,** gap weight: 12, gap length weight: 2
Consensus levels: **high=90% low=50%**
Consensus **symbols**: **!** is anyone of IV, **$** is anyone of LM, **%** is anyone of FY, § is anyone of NDQEBZ

Discussion

Investigation of the *in vivo* functionality of the three *cis* acting elements (CCAAT, XLNR, CRE) known up to now to be present in the *xyn1* promoter (Zeilinger, Mach *et al.* 1996; Wacenovsky 1998; Mach, Strauß *et al.* 1996) which are all lying in the 214 bp fragment that has been shown to contain all elements necessary for the regulation of the *xynl* gene (Mach, Strauß *et al.* 1996) worked out very well using the $xyn1_p$::*goxA* reporter gene construct. The GOX enzyme was secreted into the medium in amounts well sufficient for the activity assay (5-36 U/ml). As always the same amount of wet mycelium was used for the replacement cultures, the activity measured in the culture supernatant is related directly to the level of gene expression of the reporter gene construct in a clone and comparable among different clones. Silencing of integrated constructs was observed and led to a low but still sufficient number of clones which could be used for the expression studies (so only about 30% of the clones having the reporter gene construct integrated into the genome showed gene expression as well - not counting the clones harbouring a construct not enabling transcription due to the respective mutations). No significant difference was generally found between single- and multicopy strains, most probably being due to the inactivation of the other copies present. Only one strain harbouring two copies of the reporter gene construct seems to have both copies active for transcription (GXI-8.17). Moreover the integration locus has a clear effect on the level of transcription of the reporter gene. The effect only takes place when the transcription is not restricted to a low level by the promoter itself. When the reporter gene is expressed only on a basal level (about 5 U/ml), no influence of the integration locus is observed. Only when elevated levels of transcription are achieved, the surrounding DNA matrix and the general chromatin structure of the integration locus begins to play a role. So all reporter gene constructs leading to basal transcription level clearly group around about an activity of 5 U/ml, whereas the clones capable of inducing the transcription to a higher level show a wider variation of activities (from about 20-36 U/ml) and of induction kinetics. No significant differences in the expression levels that could be correlated to a certain mutation of a promoter element could be detected between the respective clones being capable of induction. If there were differences present, they were masked by the effect of the integration locus. But what is important, both groups (the one showing only basal expression and the one showing elevated expression) can be discriminated clearly, so that each clone can be assigned

to either group (an exception being clone GXI-2.17 which seems to have the construct integrated into a position not favourable of transcription). And enough clones were obtained to judge the effect of each mutation introduced into the *xyn1* promoter.

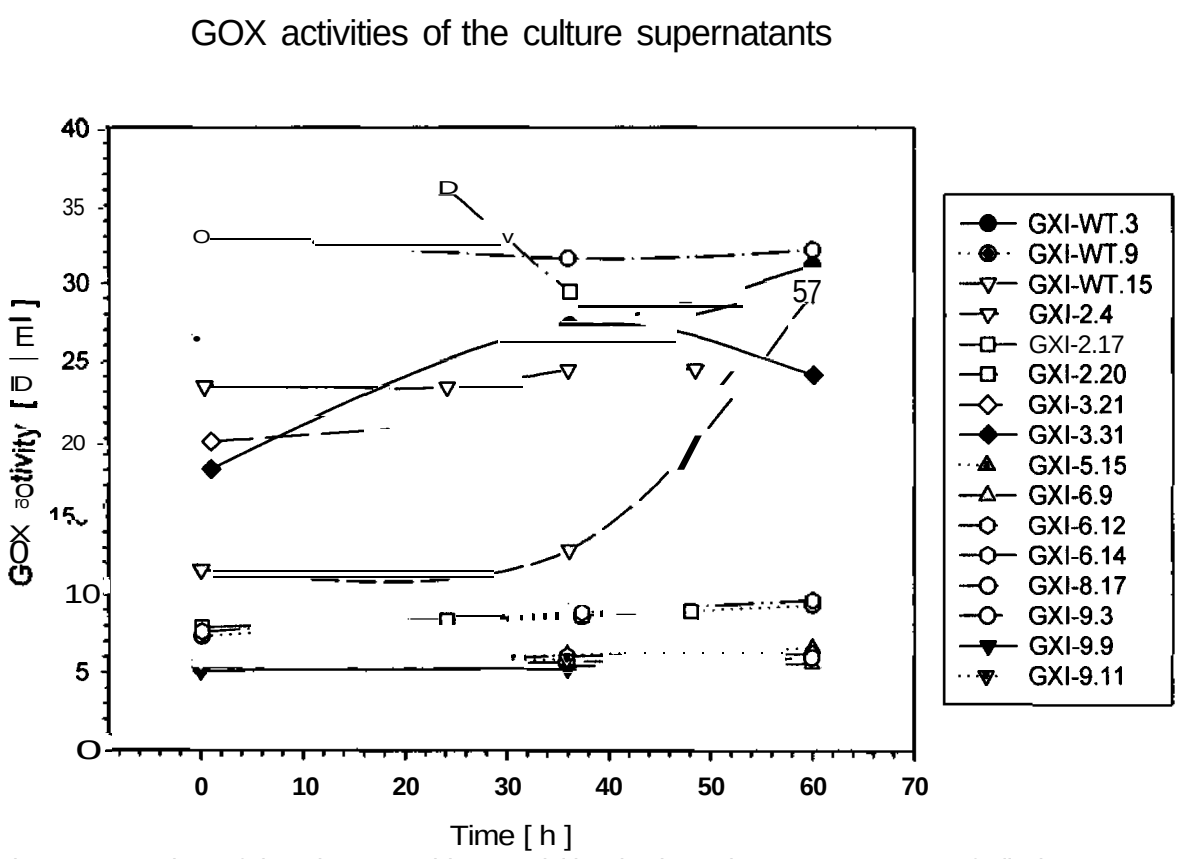

Fig. 15: Overview of the glucose oxidase activities in the culture supernatants of all clones upon induction by replacement on xylan. Clones not shown do not exhibit any detectable GOX activity.

First of all it was proven that the reporter gene construct with the wild-type promoter showed the same expression behaviour on the various carbon sources as the *xyn1* gene. It is induced by xylan and xylose and repressed by glucose and glycerol (in the expression studies glycerol was preferably used instead of glucose because it is not a substrate to glucose oxidase what otherwise could interfere with the activity measurements and the expression experiment on the whole, because the hydrogen peroxide formed by glucose oxidase can be toxic to fungi (Jacks, De Lucca *et al.* 2000)).

A deletion mutation rendering the Cre1 binding site non-functional has no effect on the level of induction but releases the gene from carbon catabolite repression leading to a basal level of transcription on glycerol. Mutating either part of the XlnR like site led clearly to a reduction (if the 5' part of the motif was concerned) or a complete loss of gene expression (if

the 3' part of the motif was altered) under inducing conditions, demonstrating that this motif and its corresponding *trans* acting factor are essential for elevated expression under inducing conditions. This is in accordance with earlier findings in EMSA experiments that alteration of the motif leads to reduced or no protein binding to this site (Wacenovsky 1998). It is not necessary for basal expression, as still a low basal level of expression can be detected even when its 3' part is mutated (XLNR-MUB) what has been shown to prevent the corresponding factor from binding. Interestingly this basal expression can be found only when the CCAAT box is not functional as well. This could lead to the assumption that the CCAAT box mediates repression of basal expression in this promoter. Although a repressor function has been shown for the CCAAT box already in other cases (Würleitner, Pera *et al.* 2002) (Weidner, Steidl *et al.* 2001), it could as well be that this effect is due to the fact that this motif and the *trans* acting HAP 2/3/5 complex binding to it act in a more general way. The expression studies presented in this work show that gene expression driven by the *xyn1* promoter is not dependent on a functional CCAAT box, as the CCTTT + CRE mutation leads to basal expression on glycerol as well as to eleveated expression comparable to the wild-type promoter on xylan. And the finding that the CRE mutation alone leads to the same expression levels on glycerol and xylan as the CCTTT + CRE mutation also shades a doubt on the role of the CCAAT box as a mere repressor. But the HAP 2/3/5 complex attached to the CCAAT box has been shown to assist in the positioning of nucleosomes in promoters, thereby influencing the accessibility of the DNA to transcriptional factors and RNA polymerase II (Narendja, Davies *et al.* 1999; Zeilinger *et al.*, manuscript submitted). A loss of function of either the CCAAT box or the HAP 2/3/5 complex leads to a loss of strictly positioned nucleosomes in these cases. So the lack of basal expression in cases where the CCAAT box remains intact (XLNR-MUB, XLNR-MUB + CRE) could be explained by nucleosomes positioned over the TATA box or the initiator region thereby preventing transcription. A finding still difficult to explain is the fact that the triple mutation (XLNR-MUB + CCTTT + CRE) does not exhibit even basal gene expression under repressing conditions, although no known functional *cis* acting element is present any more in the promoter. So there could still be some more regulatory elments. Lately it has been shown that the transcriptional repressor ACE I is involved in the regulation of the *xynl* gene (Aro, Ilmén *et al.* 2003) and that this factor can bind to the XlnR like element in this promoter (Rauscher, Würleitner *et al.*, manuscript in preparation). But as EMSA experiments indicate, the XLNR-MUB mutation abolishes binding of any protein from cell free extracts to the whole element also under conditions of repression (Wacenovsky 1998), so it seems unlikely that ACE I is responsible for this effect,

although it has to be taken into account that the conditions in an EMSA are quite different from that in the nucleus of a cell. Another possibility is given by the recent finding that Cre1 is also involved in nucleosome positioning, as the Crel negative strain RUT C-30 shows a complete loss of nucleosome arrangement in case of the *cbh2* promoter, which can be restored when a functional copy of the *cre1* gene is introduced into this strain (Zeilinger *et al.*, manuscript submitted). The *cbh2* promoter only contains single Crel binding sites and is not subjected to tight carbon catabolite repression. As there are two more single Crel sites additional to the double Crel site present in the *xyn1* promoter, these could also influence the chromatin structure in the promoter area, especially in absence of further regulatory elements providing a nucleosome free region, in a way that restrains transcription because of a tight promoter structure not allowing access by RNA polymerase II. This is in accordance with the earlier finding that a *xynl* promoter truncated at bp -100 (which also eliminates the single Crel site at bp -160) leads to the same basal activity under inducing as well as under repressing conditions (Mach, Strauß *et al.* 1996). Additionally the presence of several putative Crel binding sites in the *xyr1* promoter gives way to the assumption that the carbon catabolite repression could be tightened further by a double-lock mechanism. This hypothesis is supported by the finding, that *xynl* transcript levels in the Crel negative strain RUT C-30 are well above the level found in the wild-type strain QM9414 upon induction by xylose. Also it has been reported, that a constitutive expression of the XlnR factor can overcome carbon catabolite repression in *A. nidulans* in some cases (Orejas, MacCabe *et al.* 2001). Of course much of this is only speculative and warrants further investigations to elucidate the details of the mode of action of induction and repression of the *xynl* gene on the molecular level.

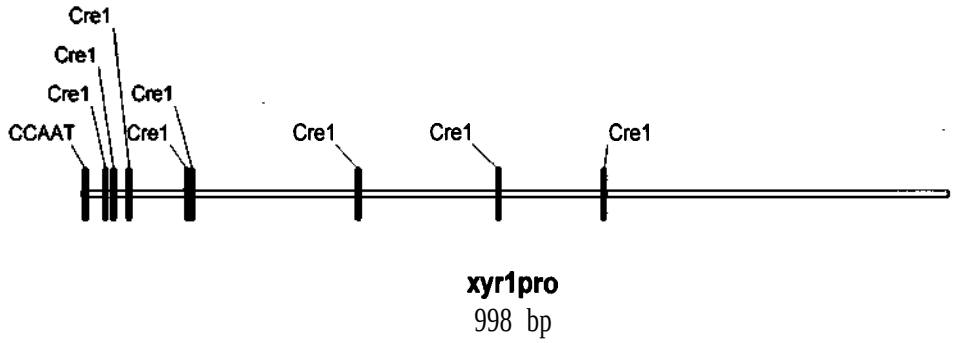

Fig 16: Putative *cis* acting elements found in the *xyr1* promoter. The CCAAT box is situated on the coding strand, all the Cre1 consensus sites are on the complementary strand.

The facts that can be derived from the expression studies are the confirmation that the Cre1 double site confers carbon catabolite repression *in vivo*, and that the XlnR like site is not only functional but absolutely essential for an elevated level of gene expression upon induction, whereas the CCAAT box although actually taking part in the regulation does not influence the level of transcription itself to a significant extent. It should be noted that for the first time expression studies were performed on the full-length *xyn1* promoter, enabling thereby influences from the interaction of the various elements and factors and from the chromatin structure to come into effect.

Furthermore a gene was cloned (*xyr1*)coding supposedly for the *trans* acting factor of the XlnR like element. This protein shows a high similarity (up to 92% identity in certain domains and an overall identity of 47,2%) to the *A. niger* XlnR factor (van Peij, Visser *et al.* 1998) which has been shown to be able to bind to the inverted GGCTAA repeat in the *xynl* promoter (Wacenovsky 1998). This together with the facts that the DNA binding zinc cluster domain as well as the C-terminus is completely conserved between XlnR and Xyr1 and that all amino acids which have been found to be indispensable for XlnR to exert its function (van Peij, Visser *et al.* 1998) are also present in Xyrl supports the assumption that Xyrl actually is the *trans* acting factor corresponding to the XlnR like double site in the *xynl* promoter.

So taking all available data together a model for the regulation can be postulated as follows. Under repressing conditions the double CRE site is occupied by Crel mediating carbon catabolite repression, the CCAAT box is occupied by the HAP 2/3/5 complex and the double XLNR motif is contacted either by a heterodimer of Xyr1/ACE I or by a homodimer of ACE I. This is deduced from the fact that it has been shown that the more general repressor ACE I (whose consensus binding sequence is GGC(T/A)AA) is able to bind to the XLNR motif as well. Furthermore one or more additional factors have to take part in the repressor complex as the EMSA with cell free extracts show that the repression specific complex migrates much slower than the induction specific complex. As ACE I has a lower molecular mass than Xyrl (whereas both have a nearly identical calculated isoelectric point, which is 6,73 for Xyrl and 6,7 for ACE I), it cannot explain the higher shift, and because the oligonucleotide fragment used in these experiments do not leave any space for further protein/DNA contact aside from the XLNR double motif, the other factor(s) are likely to bind via protein-protein interaction. Under derepressing conditions the Crel protein is released from its binding site in the promoter, leaving only the CCAAT box occupied by the HAP 2/3/5 complex and the XLNR double motif by the ACE I/Xyr1 heterodimer (respectively the ACE I homodimer). Also the additional factor(s) seem to stay bound, as only two different

sizes of complexes have been detected at the XLNR motif in the EMSA experiments - one being the large repression specific complex, the other the smaller induction specific complex. No complex of intermediate size has been found, so this assumption seems quite reasonable. When induction occurs, the ACE I repressor is replaced by Xyr1, so that beside the CCAAT box contacted by the HAP 2/3/5 complex there is now a Xyrl homodimer occupying the double XLNR motif enabling full expression of the *xyn1* gene.

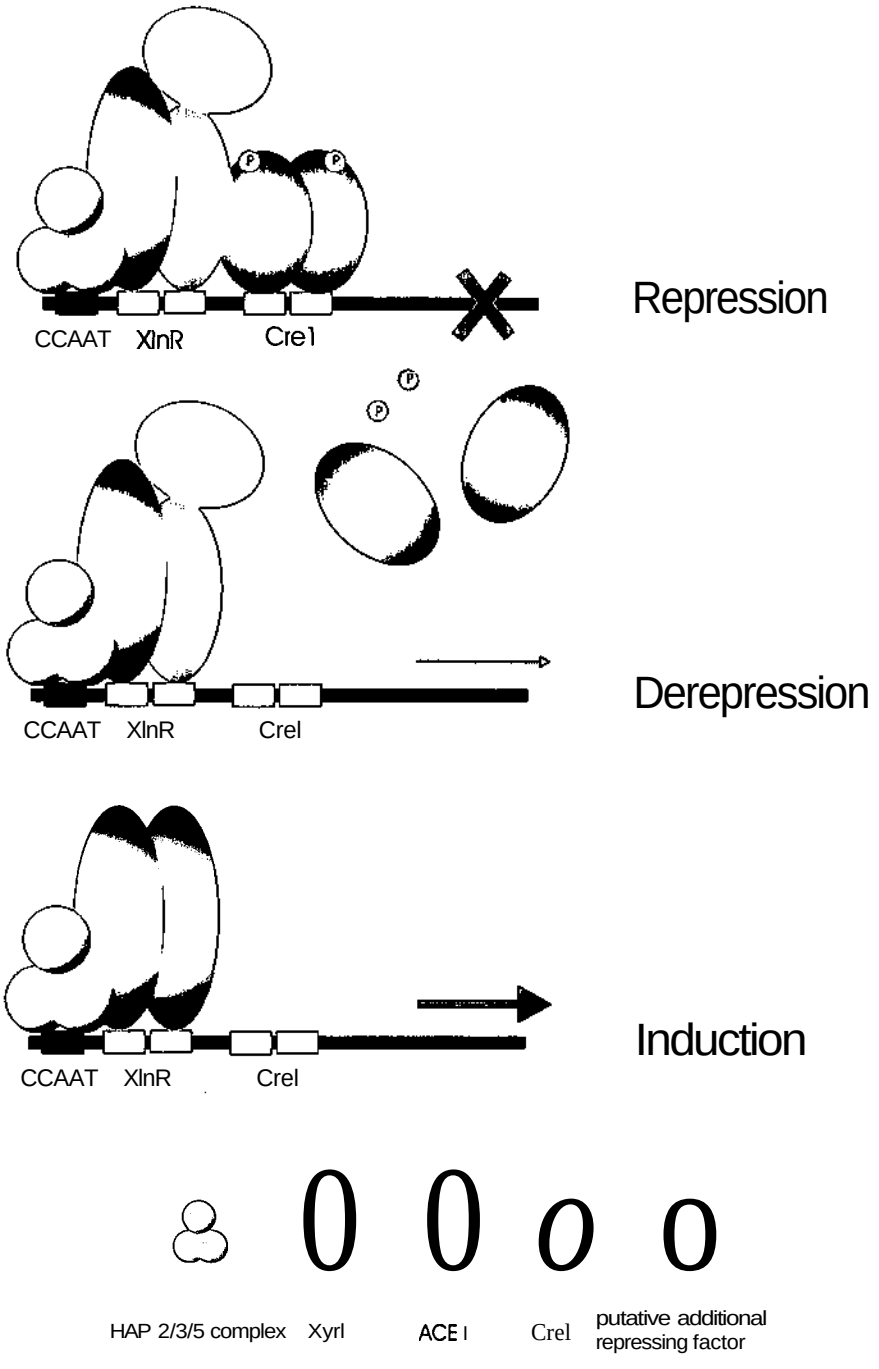

Fig. 17: Hypothetical model for the regulation of the *xyn1* gene of *Trichoderma reesei*

Summary

The industrially applied (Buchert, Oksanen *et al.* 1998; Galante, De Conti *et al.* 1998; Galante, De Conti *et al.* 1998) soft rot fungus *Trichoderma reesei* (an anamorph of *Hypocrea jecorina*) is a very efficient producer of wood degrading enzymes, the enzymatic spectrum comprising of cellulases (CBH I, CBH II, CBH III), β-glucosidases (BGL I, BGL II), endoglucanases (EG I, EG II, EG III, EG V, EG VII), xylanases (XYN I, XYN II) and several debranching enzymes (ABF I, GLR I, AXE I, AE). The fungus secretes up to 60 g/l protein upon induction of the cellulolytic system, 70% of which are cellulases, the most abundant single protein being CBH I (about 60% of the total amount of protein secreted (Fowler, Grizaldi *et al.* 1993)).

Hence the regulation of the protein production in this fungus has received much attention. But nevertheless up to now relatively little is known about how the production of extracellular proteins is controlled. Only four genes (*cbh1*, *cbh2*, *xyn1*, *xyn2*) have been investigated in some detail with regard to the processes going on at the molecular level (Stangl, Gruber *et al.* 1993; Ilmén, Onnela *et al.* 1996; Mach, Strauß *et al.* 1996; Zeilinger, Mach *et al.* 1996; Zeilinger, Mach *et al.* 1998; Zeilinger and Mach 1998; Zeilinger, Haller *et al.* 2000; Saloheimo, Aro *et al.* 2000; Aro, Saloheimo *et al.* 2001). For these depolymerising enzymes it has been shown that the control of their synthesis is exerted on the transcriptional level (Shoemaker, Schweickart *et al.* 1983; Teeri, Salovouri *et al.* 1983; Teeri, Lehtovaara *et al.* 1987; Penttilä, Lehtovaara *et al.* 1986; Saloheimo, Lehtovaara *et al.* 1988; El-Gogary, Leite *et al.* 1989; Messner and Kubicek 1991; Fowler and Brown 1992; Morawetz, Gruber *et al.* 1992; Penttilä, Saloheimo *et al.* 1993; Urnen, Onnela *et al.* 1996; Mach, Strauß *et al.* 1996; Zeilinger, Mach *et al.* 1996; Zeilinger and Mach 1998; Zeilinger, Mach *et al.* 1998; Zeilinger, Haller *et al.* 2000; Margolles-Clark, Ilmén *et al.* 1997; Würleitner, Pera *et al.* 2002).

Factors that have been found to regulate the expression of these genes and which have been cloned are Cre1 (Strauß, Mach *et al.* 1995; Ilmén, Thrane *et al.* 1996), ACE I (Saloheimo, Aro *et al.* 2000), ACE II (Aro, Saloheimo *et al.* 2001) and the HAP 2/3/5 complex (Zeilinger, Ebner *et al.* 2001). Crel and ACE I act as repressors of transcription (Mach, Strauß *et al.* 1996; Aro, Ilmén *et al.* 2003; Takashima, Iikura *et al.* 1996; Ilmén, Onnela *et al.* 1996; Strauß, Mach *et al.* 1995) whereas ACE II and in some cases also the HAP 2/3/5 promote gene expression (Aro, Saloheimo *et al.* 2001; Aro, Ilmén *et al.* 2003;

Zeilinger, Mach *et al.* 1998; Zeilinger, Ebner *et al.* 2001). In other cases the HAP 2/3/5 complex can also reduce the transcriptional activity of a gene (Würleitner, Pera *et al.* 2002). The different effects resulting from HAP 2/3/5 binding might be related to the respective overall promoter structure and the distance of the CCAAT box to the transcription start, as the complex has been shown to influence the positioning of nucleosomes (Narendja, Davies *et al.* 1999).

This work presents another novel transcriptional activator named Xyr1, a $(Zn^{2+})_2 \cdot Cys_6$ zinc cluster protein of the GAL4 type which is a homolog (47,2% identity) of the *Aspergillus niger* xylanase regulatory factor XlnR (van Peij, Visser *et al.* 1998) and indispensable for an elevated level of transcription of the *xyn1* gene of *T. reesei* upon induction. The corresponding gene has been cloned and was analysed in detail.

Also the effects of the *cis* acting elements of the *xyn1* promoter were studied *in vivo* in this work, for the first time using the full-length promoter fragment. Two motifs have already been identified earlier to regulate the transcription of the gene. A Cre1 binding site responsible for carbon catabolite repression (Mach, Strauß *et al.* 1996) and a CCAAT box recruiting the HAP 2/3/5 complex (Zeilinger, Mach *et al.* 1996; (Mach 2002) ; Rauscher, Würleitner *et al.*, manuscript in preparation). A third element exhibiting the consensus sequence of the *A. niger* XlnR binding site (GGCTAA) present in an inverted repeat was assumed to play a role in the regulation process as well (Wacenovsky 1998). By building reporter gene constructs carrying the *xyn1* promoter in front of the glucose oxidase gene (*goxA*) from *A. niger* and introducing site directed mutations into the respective elements (XLNR, CCAAT, CRE) of the promoter it could be demonstrated that the XlnR like site (XLNR) and its corresponding *trans* acting factor (which is highly likely to be Xyr1) are essential for induction of XYN I expression *in vivo* and it could be confirmed that carbon catabolite repression is mediated by the double Crel site (CRE). The CCAAT box has also been proven to take part in the regulation, but not being able to influence the transcriptional level alone to a significant extent.

Experimental procedures

Cultivation of T. reesei

T. reesei strains were grown at 30°C on 2% agar plates either with minimal medium or 3% (w/v) malt extract. When spores were used as inoculum, 0,1% peptone was added as well to facilitate germination. The strain *T. reesei* TU-6 was grown on malt extract agar supplemented with 5 mM uridine.

Liquid cultures were performed using Mandels-Andreotti medium with 1% (w/v) carbon source and 0,1% peptone. The replacement medium was Mandels-Andreotti medium with 0,5% xylan (xlyan from oat spelts, EC No. 232-760-6, contains approx. 10% arabinose, approx. 15% glucose residues; SIGMA) and no peptone. The liquid cultures were incubated at 30°C in a rotary shaker at 250 rpm.

Transformation of T. reesei *by biolistic particle bombardment*

Because there is no selectable marker present on the reporter gene construct pGXI, a selection marker was introduced using co-transformation. This marker was the *pyr4* gene from *T. reesei* QM9414 coding for orotidine-5'-phosphat decarboxylase which has been rendered non-functional in the recipient strain *T. reesei* TU-6, resulting in uridine auxotrophy. The vector carrying the *pyr4* gene was the plasmid pFG1, where a 2,7 kb *Sal* I fragment of the *T. reesei* genomic DNA, comprising the full-length gene including all up- and downstream regions necessary for transcription, was ligated into the *Sal* I site of the multiple cloning site of pGEM-5Zf(+), yielding a plasmid of 5,7 kb in total (Gruber 1990).

Preparation of the conidio spores

For the transformation the recipient strain *T. reesei* TU-6 was grown on malt extract agar containing 5 mM uridine until it had sporulated thoroughly. The conidio spores of two plates were suspended in 4 ml 0,9% NaCl / 0,01% Tween80 each and filtered over a sterile glass funnel equipped with a glass wool plug and the spore concentration was determined microscopically. A volume corresponding to 5×10^7 spores was streaked out in the middle of pyr-minimal medium agar plates and left to dry in a laminar airflow box. Five plates were used for each plasmid to be transformed plus one plate for the negative control.

Preparation of the tungsten particles

- 40-50 mg of Tungsten M-17 particles were washed three times with 1 ml of absolute ethanol, each tune spinning down the particles and discarding the supernatant.
- Then the particles were washed twice with sterile bidestilled water, each time spinning down the particles and discarding the supernatant.
- Finally the tungsten particles were suspended in 1 ml sterile bidestilled water and divided into aliquots of 50 ul. These aloquots were stored at –20°C.

Precipitation of the DNA on the tungsten particles

- First a 50 ul aliquot of tungsten particles was thawed on ice.
- Then 5 µg of pGXI as well as of pFG1 were added.
- Next 70 µl of a fresh spermidine / $CaCl_2$ solution (70 ul 0,1 M spermidine + 175 ul 2,5 M $CaCl_2$) was added and

- the suspension was mixed thoroughly for 3 minutes, followed by an
- incubation on ice for 10 minutes.
- Now the particles were spinned down briefly and the supernatant was discarded.
- 250 ul of absolute ethanol were added and the particles carefully resuspended, then
- spinning down the particles briefly again, discarding the supernatant.
- At last 50 ul of absolute ethanol were added and the particles resuspended by gently flicking the tube.
- The suspension was kept on ice until it was used.

Transformation procedure

The transformation itself was carried out using a Biolistic PDS-1000/He Particle Delivery System (BIORAD). All equipment and material used was first sterilised with 70% ethanol. Then 10 ul of the tungsten particle suspension with the DNA to be delivered into the recipient strain precipitated on them was pipetted onto each macrocarrier disk and left to dry. Finally the system was equipped with the macrocarrier disks holding the tungsten particles and the transformation procedure itself was performed operating the system according to the manufacterers instructions, using the previously prepared plates with the *T. reesei* TU-6 spores on them as a target.

The parameters of the transformation were as follows:

Rupture disks	900 psi
He pressure	1100 psi
Macrocarrier travel distance	11 mm
Gap distance	3/8" (≈ 1cm)
Microcarrier flight distance	6 cm
Air pressure	<28“ Hg

After the transformation the plates were incubated at 30°C for 3-4 days. As soon as colonies were clearly visible they were transferred to separate plates containing minimal medium agar.

Preparation of genomicDNA from T. reesei

For the preparation of genomic DNA 25 ml of malt extract medium enriched with 0,1% peptone was inoculated with 50 µl of a spore suspension (10^5 - 10^6 spores / ul) of the respective strain and incubated at 30°C on a rotary shaker for 24 hours. The mycelium was harvested using a sterile glass sinter funnel, pressed dry between two sheets of filter paper, froze in liquid nitrogen and stored at -80°C until the preparation.

- To isolate the genomic DNA the deep frozen mycelium was ground to fine powder in liquid nitrogen.
- Aliquots of the ground mycelium was added to 500 µl DNA salt solution + 400 µl equilibrated phenol and vortexed.
- The mixture was first incubated for 10 minutes at 65°C on a thermal mixer and then for another 10 minutes on ice.
- After addition of 200 ul chloroform to the suspension it was vortexed shortly, centrifuged at +4°C and 20.627×g (15.000 rpm, rotor 12154 in a Sigma 3K30 centrifuge) for 5 minutes and 400 ul of the supernatant transferred into a new tube, where 40 ul 3 M sodium acetate pH 5,3 and 1 ml of 96% ethanol were added.
- The samples were centrifuged at +4°C and 20.627×g (15.000 rpm, rotor 12154 in a Sigma 3K30 centrifuge) for 15 minutes and the supernatant discarded.
- The pellet was washed with 1 ml of 70% ethanol, centrifuged again at +4°C and 20.627×g for 10 minutes, the supernatant removed and the pellet dried at room temperature under vacuum in a SpeedVac.
- Finally the DNA was resuspended in 260 ul TE buffer + 60 ul RNase A (10 mg/ml) and stored at -20°C.

Radio labelling of DNA fragments

For plaque screening and Southern hybridisation DNA fragments intended to be used as a probe were radio labelled using random priming.

Random priming reaction mixture

8 µl template DNA
2 ul 10× priming buffer
2 µl hexamer mixture
incubate 3 min at 95°C, ½ hat RT, then 5 min on ice
3 ul dNTP mixture (without dCTP)
1,5 ul α-P^{32}-dCTP
0,5 ul Klenow fragment
incubate 30 min at 37 °C, 10 min at 68 °C
80 µl SB
put immediately on ice

- A sample of 1 ul was taken from the reaction mixture.
- Then the reaction mixture was purified chromatographically using a Sephadex G50 coloumn (1 ml Sephadex).
- Next a sample of 1 µl was taken from the eluate.
- The samples were analysed in a scintillation counter to determine the rate of incorporation of radioactive nucleotides and the overall radioactivity of the probe.
- Before use the probe was denaturated for 5 min at 95°C, then put on ice for 5 min and finally added to the hybridisation solution.

Isolation of total RNA from T. reesei

For the isolation of RNA from cultures expressing xylanase I *T. reesei* QM9414 was pregrown on Mandels-Andreotti medium containing 1% glycrol as carbon source and then replaced on Mandels-Andreotti medium with 1% xylose as sole carbon source. After eight hours of induction, the mycelium was harvested, pressed dry between sheets of filter paper and frozen in liquid nitrogen.

- The mycelium was ground to fine powder in liquid nitrogen and transferred into 2 ml tubes containing 700 ul Chirgwin reagent + 10 ul β-Mercaptoethanol resting on ice.
- Then 70 ul 3 M sodium acetate (pH 4, RNase free), 700 ul equilibrated phenol and 400 ul CI (chloroform : isoamylalcohol) were added and the mixtures incubated on ice for 15 minutes.
- After centrifugation for 20 minutes at 4°C and about 20.000xg the supernatants was transferred into new 2 ml tubes, 1 ml of isopropanol added and the RNA precipitated over night at -20°C.
- The solutions were centrifuged for 20 minutes at +4°C and about 20.000xg, the supernatant discarded and the pellet washed with 1 ml 75% ethanol (RNase free).
- Again the solutions were centrifuged for 10 minutes at +4°C and about 20.000xg, the supernatants discarded, the pellets dried at room temperature and resuspended in 100 ul bidestilled water (DEPC treated).
- Finally the concentration of RNA present in the preparations was determined spectrophotometrically. The concentrations obtained were between 4 µg/µl and 8 µg/µl, the total amount of RNA yielded was 9,4 mg.

Preparation of mRNA from total RNA

For the preparation of mRNA the total RNA isolated from *T. reesei* QM9414 mycelium before was used. For the separation of mRNA from other RNA species a PolyATtrackt® kit (PROMEGA) was used. The principle of this purification procedure is that the poly-A tail of mRNA associates with an oligo(dT) probe coupled to paramagnetic particles via streptavidin / biotin interaction and can be collected using a magnetic stand, while the rest of the RNA is washed out.

- About 1 mg of total RNA (~200 ul) were brought to a final volume of 500 ul in RNase free water.
- The tube was placed in a heating block at 65°C for 10 minutes.
- 3 ul of biotinylated oligo(dT) probe and 13 ul of 20 x SSC were added, mixed gently and incubated at room temperature until completely cooled.
- The streptavidin coated paramagnetic particles were dispersed by flicking the tube, collected by placing the tube in the magnetic stand, the supernatant removed, and the particles washed three times with 300 ul 0,5× SSC. Afterwards the particles are suspended in 100 ul 0,5 x SSC.
- Now the entire annealing reaction containing the now biotin labelled mRNA was added to the tube holding the suspended paramagnetic particles and the mixture was incubated at room temperature for 10 minutes.
- The paramagnetic particles were then captured using the magnetic stand, the supernatant was removed and the particles were washed four times with 300 ul of 0,1 x SSC.
- Finally the mRNA was released by adding 100 ul of RNase free water, resuspending the paramagnetic particles, capturing them again using the magnetic stand and transferring the supernatant to a sterile RNase free tube. To increase yield this step was repeated with another 150 ul of RNase free water, combining the eluates to a total volume of 250 ul containing the purified mRNA. The amount of mRNA recovered was 25,2 µg, that is 0,27% of the amount of total RNA which was started with.

Construction of a T. reesei *cDNA library (HYBRIZAP®-2.1)*

For reverse transcription the mRNA had to be concentrated, so it was precipitated using 0,15 volumes of 3 M sodium acetaet and one volume of isopropanol, incubating the mixture at -20°C over night and centrifuging it at +4°C and 20.000xg for 30 minutes, discarding the supernatant, washing the pellet with 2 ml of 75% RNase free ethanol, centrifuging again at +4°C and 20.000×g for 10 minutes, removing the supernatant, drying the pellet at room temperature and resuspending it in 15 µl of RNase free water, now having a mRNA concentration of 0,84 µg/µl.

The cDNA library was prepared according to the manufacterer's instructions (manual #235612-12, STRATAGENE) using about 5 µg (7 µl) of mRNA, yielding 120 ng of cDNA which was ligated into the HybriZAP®-2.1 vector and packaged into phage particles. This unamplified cDNA library showed a titer of 5×10^6 pfu/ml. After amplification using *E. coli* XL1 -BLUE MRF' as host strain the final cDNA library reached a titer of 8×10^9 pfu/µl.

Glucose oxidase (GOX) assay

Glucose oxidase converts glucose to glucono-δ-lactone thereby releasing hydrogen peroxide (H_2O_2). So the glucose oxidase activity present in a sample can be determined by the amount of hydrogen peroxide formed during a certain period of time. As it is difficult to detect the hydroxide peroxide directly, horse radish peroxidase is included in the assay, which can use hydrogen peroxide to oxidise certain substrates. When the colour of the oxidised and reduced state of such a substrate differs, the increase of the concentration of oxidised substrate can be measured spectrophotometrically and correlated to glucose oxidase activity. A compound used frequently in this context is 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), which is nearly colourless in the reduced state and green in the oxidised state.

For glucose oxidase assay 1 ml of culture broth was taken and centrifuged at +4°C and ~20.000xg for 10 minutes. The supernatant was then transferred into a new tube and stored at -20°C.

Reaction mixture:
400 ul mM ABTS solution (1,25 mM)
400 ul glucose solution (630 mM)
100 ul horse radish peroxidase solution (10 U/ml)
(all solutions prepared in 111 mM NaH_2PO_4 buffer pH 5,8)

The solutions were combined in a plastic cuvette and placed in the photometer connected to a plotter. The absorbance at 420 nm was monitored and when a stable reading was reached the reaction was started by adding 100 µl of the sample solution directly into the cuvette. The following increase of the absorbance was recorded by the plotter and evaluated.

1 unit [U] is the amount of glucose oxidase, which oxidises 1 µmol glucose within 1 minute at 25°C at a pH of 5,8.

So 1 U glucose oxidase produces 1 µmol H_2O_2 in 1 minute. Thus a calibration curve plotting known amounts of H_2O_2 versus the absorbance at 420 nm after the reaction with ABTS catalysed by HRP gives a correlation between the absorbance observed and the activity of glucose oxidase present in a sample.

c (H_2O_2) [µM]	Absorbance (A_{420})
0,78	0,04
1,56	0,09
3,13	0,15
6,25	0,28
12,50	0,77

Table 8: Calibration values for the glucose oxidase assay

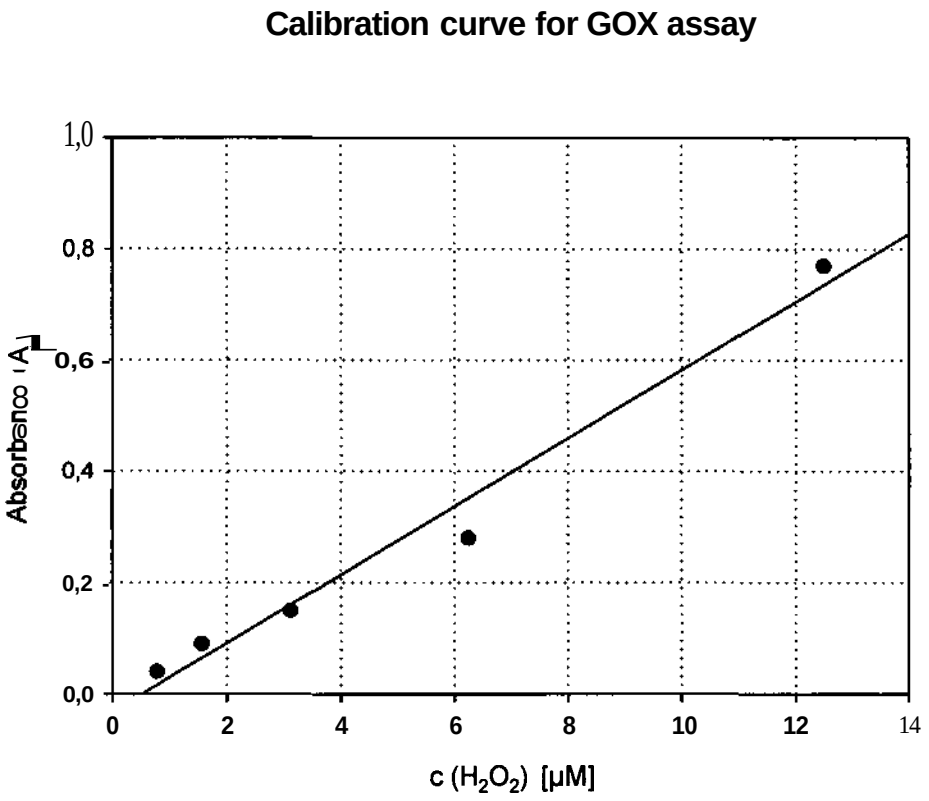

Fig. 18: Calibration curve for the glucose oxidase assay

Transformation of E. coli

For transformation of plasmids and ligation reactions always chemically competent cells were used and the procedure performed as below:

- Thawing of an aliquot of 50 ul of chemically competent *E. coli* on ice.
- Adding 1 ul of DNA in an apt dilution or 10 ul of a ligation reaction.
- Incubation on ice for 20 minutes.
- Heat shock at 42°C for 30 seconds.
- Adding of 800 ul LB medium.
- " Incubation at 37°C for 40 minutes.
- Spinning down the cells using a Sigma 113 centrifuge at room temperature and 13.000 rpm for 1 minute.
- Discarding 750 ul of the supernatant.
- Resuspending the pellet in the remaining supernatant.
- Plating the cell suspension on selective plates, 50 ul on each.
- Incubation of the plates over night at 37°C.

QIAEX gel extraction protocol

as performed practically

- Excise the DNA band from the agarose gel and transfer it into a 1,5 ml tube.
- Weigh the gel slice and add 300 ul of Buffer QX1 to each 100 mg of gel.
- Resuspend the QIAEX II suspension by vortexing for 30 seconds and add 10 ul of the suspension to the gel slice.
- Incubate at 50°C for 10 minutes to solubilize the agarose and bind the DNA. Mix by vortexing every 2 minutes to keep the QIAEX II particles in suspension.
- Centrifuge the sample for 30 seconds and carefully remove the supernatant with a pipet.
- Wash the pellet with 500 µl of Buffer QX1.
- Wash the pellet twice with 500 ul of Buffer PE.
- Air-dry the pellet until it becomes white.
- Elute the DNA by adding 20 µl sterile bidestilled water, resuspending the pellet by vortexing and incubating the suspension at 50°C for 10 minutes.
- Centrifuge for 30 seconds. Carefully pipet the supernatant into a clean tube.

Standard plasmid preparation protocol

- *E. coli* grown over night in 3 ml LB medium containing an appropriate antibiotic were harvested by centrifugation 1 ml of the cell suspension for 10 minutes at 20.627×g (15.000 rpm in a Sigma 3K30 centrifuge using a 12154 rotor) and +4°C.
- The bacterial pellet was resuspended in 250 ul buffer P1.
- 250 ul of buffer P2 were added, the content of the tube mixed gently and incubatet at room temperature for 5 minutes.
- Then 250 ul of buffer P3 were added and the mixture incubatetd on ice for 10 minutes.
- The cell debris, protein and chromosomal DNA were removed by centrifugation for 10 minutes at 20.627×g and +4°C.
- The plasmid DNA was then precipitated by adding 700 ul isopropanol.
- After centrifugation for 30 minutes at 20.627×g and 4°C the supernatant was discarded, the pellet washed with 1 ml 70% ethanol, centrifuged again for 10 minutes at 20.627×g and +4°C, the supernatant discarded, the pellet dried in a SpeedVac under vacuum at room temperature and finally resuspended in 20 ul sterile bidestilled water.

QIAGEN mini plasmid preparation protocol

as performed practically

- *E. coli* grown over night in 3 ml LB medium containing an appropriate antibiotic were harvested by centrifugation 1 ml of the cell suspension for 10 minutes at 20.627×g (15.000 rpm in a Sigma 3K30 centrifuge using a 12154 rotor) and +4°C.
- The bacterial pellet was resuspended in 250 ul buffer P1 plus 5 ul RNase A (10 mg/ml).
- 250 ul of buffer P2 were added, the content of the tube mixed gently and incubatet at room temperature for 5 minutes.
- Then 250 µl of buffer P3 were added and the mixture incubatetd on ice for 10 minutes.
- The cell debris, protein and chromosomal DNA were removed by centrifugation for 10 minutes at 20.627×g and +4°C.
- The supernatant was applied to a QIAGEN-tip20 purification coloumn which was equilibrated before using 1 ml of buffer QBT.
- When the supernatant had entered the resin of the coloumn completely, the coloumn was washed four times with 1 ml of buffer QC.
- The plasmid DNA was then eluted from the coloumn with 800 ul of buffer QF and precipitated by adding 700 ul isopropanol.
- After centrifugation for 30 minutes at 20.627×g and 4°C the supernatant was discarded, the pellet washed with 1 ml 70% ethanol, centrifuged again for 10 minutes at 20.627xg and +4°C, the supernatant discarded, the pellet dried in a SpeedVac under vacuum at room temperature and finally resuspended in 20 ul sterile bidestilled water.

Media

Malt extract agar

3% (w/v) malt extract
2% (w/v) agar agar

LB medium

10 g/l NaCl
5 g/l yeast extract
10 g/l peptone

pH 7,2-7,5

LB/Amp

10 g/l NaCl
5 g/l yeast extract
10 g/l peptone
100 mg/l ampicillin

pH 7,2-7,5

LB/Amp agar

10 g/l NaCl
5 g/l yeast extract
10 g/l peptone
1,5% agar agar
100 mg/l ampicillin

pH 7,2-7,5

LB agarose bottom

10 g/l	NaCl
5 g/l	yeast extract
10 g/l	casein hydrolysate
1,5%	agarose

Top agarose

5 g/l	NaCl
10 g/l	casein hydrolysate
0,6%	agarose

Minimal medium agar

1 g/l	$MgSO_4 \cdot 7\,H_2O$
6 g/l	$(NH_4)_2SO_4$
1 g/l	KH_2PO_4
3 g/l	Na_3 citrate$\cdot 2\,H_2O$
10 g/l	glucose
20 ml	50× trace element solution
1,5%	agar agar

pH 5,8

Mandels-Andreotti medium

500 ml/l	mineral salt solution
480 ml/l	0,1 M phosphate citrate buffer pH 5
20 ml/l	50× trace element solution
10 g/l	carbon source
1 g/l	peptone
0,3 g/l	urea (5 mM)

Solutions

Mineral salt solution

2,8 g/l $(NH_4)_2SO_4$ (21,19 mM)
4,0 g/l KH_2PO_4 (29,16 mM)
0,6 g/l $MgSO_4 \cdot 7\ H_2O$ (2,43 mM)
0,8 g/l $CaCl_2 \cdot 2\ H_2O$ (5,44 mM)

50× Trace element solution

900 µM $FeSO_4 \cdot 7\ H_2O$
310 µM $MnSO_4 \cdot H_2O$
240 uM $ZnSO_4 \cdot 7\ H_2O$
680 µM $CaCl_2 \cdot 2\ H_2O$

SM buffer

0,1 M NaCl
8 mM $MgSO_4 \cdot 7\ H_2O$
50 mM Tris
0,1 g/l gelatin

pH 7,5 (HC1)

Hybridisation solution

10 % (v/v) 50x Denhardt's reagent
30 % (v/v) 20× SSC
0,5 % (w/v) SDS
100 µg/ml ssDNA (salmon sperm DNA)

50× Denhardt's reagent

1 % (w/v) Ficoll
1 % (w/v) polyvinylpyrrolidone
1 % (w/v) BSA

10× Priming buffer

500 mM Tris
100 mM $MgCl_2 \cdot 6\ H_2O$

pH 7,5

NaH_2PO_4 buffer

111 mM NaH_2PO_4

pH 5,8 (NaOH)

ABTS solution for GOX assay

1,25 mM ABTS
1,25 mM NaN_3
111 mM NaH_2PO_4

pH 5,8 (NaOH)

Glucose solution for GOX assay

630 mM glucose
111 mM NaH_2PO_4

pH 5,8 (NaOH)

Horse radish peroxidase solution for GOX assay

10 U/ml horse radish peroxidase
111 mM NaH_2PO_4

pH 5,8 (NaOH)

DNA salt solution

0,1 M Tris
5 mM EDTA
1 mM DTT
1,2 M NaCl

pH 8,0 (HCl)

TE buffer

10 mM Tris
1 mM EDTA

pH 8,0 (HC1)

P1 (resuspension buffer)

100 µg/ml RNase A
50 mM Tris
10 mM EDTA·2 H_2O

pH 8,0 (HC1)

P2 (lysis buffer)

200 mM NaOH
1 % (w/v) SDS

P3 (neutralisation buffer)

3,0 M potassium acetate

pH 5,5 (acetic acid)

QBT (equilibration buffer)

750 mM NaCl
50 mM MOPS
15 % (v/v) EtOH (96 %)
0,15 % (w/v) Triton X-100

pH 7,0

QC (washing buffer)

1,0 M NaCl
50 mM MOPS
15 % (v/v) EtOH (96 %)

pH 7,0

QF (elution buffer)

1,25 M NaCl
50 mM Tris
15 % (v/v) EtOH (96 %)

pH 8,5 (HC1)

Abbreviations

aa	amino acid(s)
ABF	arabinofuranosidase
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ADP	adenosindiphosphate
AE	acetyl esterase
Amp	ampicillin
ATP	adenosintriphosphate
AXE	acetyl-xylanesterase
BGL	β-glucosidase
bp	base pair(s)
BSA	bovine serum albumine
BXL	β-xylosidase
CAE	*cbh2* activating element
CBD	cellulose bindending domain
CBD	cellulose binding domain
CBH	cellobiohydrolase
cDNA	complementary DNA
CDP	CCAAT displacement protein
CTP	cytidintriphosphat
DEPC	diethylpyrocarbonate
DNA	desoxyribonucleic acid
dNTP	2'-desoxynucleotidetriphosphate
dsDNA	double stranded DNA

E.C.	enzyme comission
EDTA	ethylendiammintetraacetate
EG	endoglucanase
EMSA	electophoretic mobility shift assay
EST	expressed sequence tag
Glc	glucose
GlcNAc	N-acetylglucosamine
GLR	glucuronidase
GOX	glucose oxidase
GTP	guanosintriphosphate
HFM	histone fold motif
hnRNA	heterogenous nuclear RNA
Inr	initiator region
IPTG	isopropyl-beta-D-thiogalactopyranoside
LD	lethal dose
Man	mannose
MEX	malt extract
MOPS	3-(N-morpholino) propane sulphonic acid
mRNA	messenger RNA
nfr	nucleosome free region
nt	nucleotide(s)
NTP	nucleotidetriphosphate
o/n	over night
ORF	open reading frame
PCR	polymerase chain reaction
PEG	polyethylenglykol

Pol II	RNA polymerase II
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	room temperature
RT PCR	reverse transcription PCR
SB	sterile bidestilled H_2O
SDS	sodiumdodecylsulphate
SP1	specifity protein 1
ssDNA	single stranded DNA
TAF	TATA binding protein associated factor
TBP	TATA-bindendes Protein
TF	transcription factor
Tris	Tris(hydroxymethyl)aminomethane
XAE	*xyn2* activating element
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
XYN	xylanase

Nucleotide bases

Abbreviation	Base
A	adenin
B	C, G or T
C	cytosin
D	A, G or T
G	guanin
H	A, C or T
K	G or T
M	A or C
N	A, C, G or T
R	A or G
S	G or C
T	thymin (DNA)
U	uracil (RNA)
V	A, C or G
W	A or T
Y	C or T

Amino acids

Amino acids		
One-letter code	Three-letter code	Name
A	Ala	alanin
C	Cys	cystein
D	Asp	aspartic acid
E	Glu	glutamic acid
F	Phe	phenylalanin
G	Gly	glycin
H	His	histidin
I	Ile	isoleucin
K	Lys	lysin
L	Leu	leucin
M	Met	methionin
N	Asn	asparagin
P	Pro	prolin
Q	Gln	glutamin
R	Arg	arginin
S	Ser	serin
T	Thr	threonin
V	Val	valin
W	Trp	tryptophan
Y	Tyr	tyrosin

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1998	Graduation as Diplom-Ingenieur with distinction
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