(54) Titre : SECRETION DE PROTEINES AMELIOREE DANS DES CELLULES EUCARYOTES
(54) Title: IMPROVED PROTEIN SECRETION IN EUKARYOTIC CELLS

(57) Abrégé/Abstract:
The present invention relates to the use of a glucosidase II mutation to increase protein secretion in eukaryotic cells. The present invention relates further to the use of an eukaryotic cells, comprising a mutant glucosidase II gene, possibly in combination with the expression of a recombinant α-1,2 mannosidase gene and/or a recombinant N-acetylglucosaminyl-transferase gene, as a host for protein secretion.
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Abstract: The present invention relates to the use of a glucosidase II mutation to increase protein secretion in eukaryotic cells. The present invention relates further to the use of an eukaryotic cells, comprising a mutant glucosidase II gene, possibly in combination with the expression of a recombinant α-1,2 mannosidase gene and/or a recombinant N-acetylglucosaminyl-transferase gene, as a host for protein secretion.
IMPROVED PROTEIN SECRETION IN EUKARYOTIC CELLS

The present invention relates to the use of a glucosidase II mutation to increase protein secretion in eukaryotic cells.

The present invention relates further to the use of an eukaryotic cells, comprising a mutant and/or recombinant glucosidase II gene, possibly in combination with the expression of a recombinant α-1,2-mannosidase gene and/or a recombinant N-acetylglucosaminyl-transferase gene, as a host for protein secretion.

Filamentous fungi produce high yields of proteins and metabolites. Impressive increases in the secretion of homologous proteins were obtained with traditional strain-improvement strategies based on various mutagenesis approaches. As such, industrial strains have been created which secrete >20g/l of a specific endogenous protein. In this way, filamentous fungi seem promising organisms for the production of heterologous proteins of biomedical interest (Muras et al., 1999; Punt et al., 2002).

However, contrary to mammalian cells, these lower eukaryotic organisms are not synthesizing complex type protein-linked oligosaccharides. This hampers the use of therapeutic glycoproteins produced by filamentous fungi, since they mostly synthesize high-mannose type N-glycans. Due to the presence of several lectins on human cells, glycoproteins carrying this type of glycosylation are rapidly cleared from the blood stream. This significantly reduces their therapeutic value.

Not only are lower eukaryotes like filamentous fungi, unable to synthesize complex type oligosaccharides, they sometimes also elongate the high-mannose type glycans with fungal-specific glycan residues like mannostephamphate, α-1,3-mannose and galactofuranose. Some of these residues induce an immunogenic response in humans, again reducing the therapeutic value of such glycoproteins.

Protein N-glycosylation originates in the endoplasmic reticulum (ER), where an N-linked oligosaccharide (GlcNAc-Manα1,3GlcNAc2) assembled on dolichol (a lipid carrier intermediate) is transferred to the appropriate Asn of a nascent protein. This is a co-translational event common to all eukaryotic organisms. The three glucose residues and one specific α-1,2-linked mannose residue are removed by specific glucosidases and an α-1,2-mannosidase in the ER, resulting in the core oligosaccharide structure, Manα1,3GlcNAc2. Proteins with this core sugar structure are transported to the Golgi apparatus where the sugar moiety undergoes various modifications. There are significant differences in the modifications of the sugar chain in the Golgi apparatus between lower and higher eukaryotes.

In mammalian cells, the modification of the sugar chain can follow 3 different pathways depending on the protein moiety to which it is added. That is: (1) the core sugar chain does not change; (2) the core sugar chain is changed by adding the N-acetylglucosamine-1-phosphate moiety (GlcNAc1-P) in UDP-N-acetyl glucosamine
(UDP-GloNAc) to the 6-position of mannose in the core sugar chain, followed by removal of the GlcNAc moiety to form an acidic sugar chain in the glycoprotein; and (3) the core sugar chain is first converted into Man\textsubscript{9}GlcNAc\textsubscript{2} by removing 3 mannose residues with Golgi α-Mannosidase I; Man\textsubscript{5}GlcNAc\textsubscript{2} is then further modified by adding GlcNAc and removing 2 more mannose residues, followed by sequentially adding GlcNAc, galactose (Gal), and N-acetylneuraminic acid (also called sialic acid (NeuNAc)) to form various hybrid or complex sugar chains (R. Kornfeld and S. Kornfeld, 1985; Chiba et al., 1998).

In filamentous fungi like *Trichoderma reesei*, only a part of the Man\textsubscript{9}GlcNAc\textsubscript{2} structures are (partially) trimmed down to Man\textsubscript{7}GlcNAc\textsubscript{2}. These oligosaccharides can then be further modified to fungal-specific glycans through the addition of mannosephosphate residues in a diester linkage. As such, a variety of sugar residues can be found on *Trichoderma* secreted glycoproteins, consisting of Man\textsubscript{5}GlcNAc\textsubscript{2} with or without one or two mannosephosphate residues. An exception to this general *Trichoderma* glycosylation pattern is the Rut-C30 strain, producing mainly GlcMan\textsubscript{7}GlcNAc\textsubscript{2} or GlcMan\textsubscript{7}GlcNAc\textsubscript{2}-P-Man (Maras et al., 1997).

There is a clear need for a fungal strain, such as a *Trichoderma* strain, that is able to secrete large amounts of a heterologous protein with a more human-compatible glycosylation profile. As such, the Rut-C30 strain of *Trichoderma reesei* which is a hypersecretor of endogenous cellulases (up to 30 g/l), would be an interesting strain for heterologous protein production, but it is hampered by its aberrant glycosylation pattern, compared to the wild type Qm6a strain and to most of the industrial mutant strains. In these *Trichoderma* strains, a first α-1,2-linked glucose residue is removed by glucosidase I, after transfer of the Glc\textsubscript{3}Man\textsubscript{7}GlcNAc\textsubscript{2} structure to the protein. This is followed by the removal of the two α-1,3-linked glucose residues by glucosidase II. However in the Rut-C30 strain, NMR analysis revealed that more than 80% of the glycan structures synthesized on cellobiohydrolase I (CBH I) still contained one α-1,3-linked glucose residue at the end of the α-1,3-arm of the high-mannose core structure (Maras et al, 1997). This indicates a malfunction at the level of the glucosidase II. This malfunction could be due to a reduced expression level of the enzyme. However, surprisingly we found that this malfunction is due to the presence of a frameshift mutation within the Rut-C30 glucosidase II ORF, presumably deleting or severely damaging the Glc-α-1,3-Man substrate binding site, but not the Glc-α-1,3-Glc substrate binding site. This presumption would be in accordance with the kinetic model proposed by Alonso et al. (1993), in which the two substrate binding sites are proposed, and could also explain why the removal of the first α-1,3-linked glucose residue does not seem to present any problem.

Even more surprisingly we found that a Rut-C30 strain expressing a fully functional (ER-localized) *Trichoderma reesei* glucosidase II was showing a changed glycosylation profile, resembling that of most other *Trichoderma reesei* strains. However, the secretion level was affected by the expression of the glucosidase II. Coexpression of glucosidase II, α-1,2-
mannosidase and GlcNac-transferase resulted in a modified secretion, combined with a human-like glycosylation profile. The resulting strain may be useful for the production of heterologous proteins of which the glycosylation pattern is critical.

Knocking out the glucosidase II gene in Saccharomyces cerevisiae, as well as the introduction of the mutant glucosidase II form similar to the Trichoderma reesi RUT C30 mutation confirms the unexpected effect of the glucosidase II mutation on the protein secretion. Therefore, a first aspect of the invention is the use of a glucosidase II mutation to increase protein secretion in eukaryotic cells. Every mutation that affects the activity of the glucosidase II may be used, and it may be, as a non-limiting example, an inactivating or downregulating mutation in the promoter region, an inactivating knock out of a part of the coding sequence or of the whole coding sequence, a point mutation in one or more of the subunits of the glucosidase II, or an exchange of one or more of the subunits by a mutant subunit or by a subunit of another species. Preferably, the effect of the mutation is a decrease in activity of glucosidase II. Preferably, the subunit that carries the mutation is subunit alpha.

The eukaryotic cells may be any eukaryotic cells, including, but not limited to mammalian cells, insect cells, plant cells and fungal cells. Preferably, said eukaryotic cell is a fungal cell, even more preferably a filamentous fungus or a yeast cell. Filamentous fungi are known to the person, skilled in the art, and include, but are not limited to, species from the genera Aspergillus, Fusarium, Geotrichum, Monascus, Monilia, Mucor, Penicillium, Rhizopus, Trichoderma and Ustilago. Preferably, said filamentous fungus is a Trichoderma sp., even more preferably said filamentous fungus is Trichoderma reesi Rut-C30. Yeast cells are also known to the person skilled in the art and include, but are not limited to Saccharomyces sp., Pichia sp., Hansenula sp., Kluyveromyces sp. and Schizosaccharomyces pombe. Preferably, said yeast cell is a Saccharomyces cerevisiae strain.

The secreted proteins may be homologous proteins or heterologous proteins, and they may be glycosylated or not glycosylated. Preferably the secreted proteins are heterologous proteins, and even more preferably, the proteins are glycosylated heterologous proteins. Another aspect of the invention is the use of a recombinant filamentous fungus comprising a defective recombinant glucosidase II as a host for protein secretion.

A defective recombinant glucosidase II as used here means that the endogenous sequence of the promoter and/or of the coding sequence of one or more of the subunits of glucosidase II has been replaced by a non-endogenous sequence. Preferably, the subunit that is replaced is subunit alpha. Said non-endogenous sequence may be the sequence of a non-glucosidase II gene of the same organism, or the sequence of another organism, or an artificial sequence. The resulting defective recombinant glucosidase II should have an activity that is different from the wild type, preferably a lower activity. Filamentous fungi are known to the person, skilled in the art, and include, but are not limited to, species from the genera Aspergillus, Fusarium,
Geotrichum, Monascus, Monilia, Mucor, Penicillium, Rhizopus, Trichoderma and Ustilago. Preferably, said filamentous fungus is a Trichoderma sp., even more preferably said filamentous fungus is Trichoderma reesei Rut-C30.

Protein secretion as used here may be the secretion of an endogenous protein, or the secretion of a heterologous protein.

Still another aspect of the invention is the use of a yeast comprising a defective glucosidase II as a host for protein secretion. The defective glucosidase II has an activity that is different from the wild type, preferably a lower activity. Said defective glucosidase II might be obtained by random mutagenesis. However, preferably said defective glucosidase II is a defective recombinant glucosidase II, as discussed above. Yeast cells are preferably selected from the group consisting of Saccharomyces sp., Pichia sp., Hansenula sp., Kluyveromyces sp. and Schizosaccharomyces pombe. Even more preferably, said yeast cell is a Saccharomyces cerevisiae strain.

Still another aspect of the invention is a method to increase protein secretion of a eukaryotic cell, comprising mutagenesis of glucosidase II. Techniques for mutagenesis are known to the person skilled in the art, and include, but are not limited to chemical mutagenesis, physical mutagenesis such as UV radiation, or site directed mutagenesis by recombinant DNA techniques. Preferably, said mutagenesis is site directed mutagenesis. Preferably, said eukaryotic cell is a fungal cell, such as a filamentous fungus or a yeast cell. Glucosidase II genes have been cloned from a number of mammalian species including rat (Trombetta et al., 1996), mouse (Arendt et al., 1997), pig (Flura et al., 1997) and human (Trombetta et al., 1996, genbank accession number D42041). The glucosidase II protein from these mammalian species consists of an alpha and a beta subunit. The alpha subunit is about 110 kDa and contains the catalytic activity of the enzyme, while the beta subunit has a C-terminal HDEL ER-retention sequence and is believed to be required for the ER localization of the enzyme. Similar results were obtained for the fission yeast S. pombe (d'Alessio et al., 1999). The sequence of the glucosidase II gene from S. cerevisiae has also been identified (ORF YBR229c, located on chromosome II, genbank accession number Z36098). This gene encodes a protein of about 110 kDa, which shows a high degree of homology to the mammalian alpha subunits. During the course of our work, the genes coding for the a-subunits of the Trichoderma reesei Rut-C30 and the Aspergillus niger glucosidase II protein, were cloned, facilitating the site directed mutagenesis of the genes. Transformation vectors and transformation techniques for yeast and filamentous fungi are known to the person skilled in the art. For Trichoderma, preferred vectors carrying a glucosidase II expression sequence are called pFGPDglsIITreesei and pFGPDglsIITreesei(Myc).
Vectors can be introduced into the cells of a *Trichoderma* strain using known methods such as the protoplast technique, described by Penttila et al., 1987. Other published methods useful for transformation of the plasmids or linear vectors include electroporation (Goldman et al., 1990), particle bombardment (Lorito et al., 1993) and an *Agrobacterium tumefaciens*-mediated strategy (de Groot et al., 1998).

During the transformation procedure, the glucosidase II expression sequence is cotransformed with a selection plasmid. By 'selection plasmid' is meant a plasmid carrying a selection marker. By 'selection marker' is meant an expression cassette coding for a specific gene product, which enables us to discriminate between a transformed strain and a non-transformed strain. Transformed *Trichoderma* clones can be selected by using appropriate techniques including but not limited to culturing auxotrophic cells after transformation in the absence of the biochemical product required (due to the cell's auxotrophy), selection for and detection of a new phenotype, or culturing in the presence of an antibiotic which is toxic to the fungus in the absence of a resistance gene within the transformants. Examples of available selection markers for *Trichoderma reesei* are the acetamidase expression cassette of the vector p3SR2 (Hynes et al., 1983) (enabling transformed strains to grow on acetamide as a sole nitrogen source), the *E. coli* hygromycin B phosphotransferase cassette of vector pAN7.1 (Punt et al., 1987) and the *Streptococcus* hindustanus phleomycin-binding protein expression cassette of vector pAN8.1 (Mattern et al., 1998) (enabling the transformed strains to grow on a certain concentration of hygromycin resp. phleomycin).

Another aspect of the invention is a genetically engineered filamentous fungus expressing a glucosidase II gene according to the invention, further expressing a recombinant α-1,2-mannosidase gene. Preferably, said α-1,2-mannosidase gene is fused to an ER retention signal. More preferably, said ER retention signal is derived from the MNS1 protein of *S. cerevisiae*. Even more preferably, said retention signal comprises the sequence HDEL. Preferably, said filamentous fungus is a *Trichoderma* sp., even more preferably said filamentous fungus is *Trichoderma reesei* Rut-C30.

An α-1,2-mannosidase cleaves the α-1,2-linked mannose residues at the end of Man₉GlcNAC₂, and converts this core oligosaccharide on glycoproteins to Man₉GlcNAC₂ which is thought to be a very poor substrate for a Golgi phosphomannosyltransferase. Thus, by introducing an α-1,2-mannosidase into filamentous fungi such as *Trichoderma*, glycoproteins with reduced mannose and phosphate content can be produced. Furthermore, Man₉GlcNAC₂ is the acceptor substrate for the mammalian N-acetylgalactosaminyl transferase I and as such a key structure in the synthesis of hybrid- and complex-type sugar chains, characteristic for mammalian glycoproteins.
According to the present invention, a genetically engineered *Trichoderma* strain capable of expressing an α-1,2-mannosidase can be generated by introducing into the filamentous fungus a nucleotide sequence capable of expressing the α-1,2-mannosidase.

According to the present invention, the nucleotide sequence encoding an α-1,2-mannosidase for introduction into a *Trichoderma* strain can derive from any species. A number of α-1,2-mannosidase genes have been cloned from different species and are available to those skilled in the art, including mammalian genes encoding, e.g., a murine α-1,2-mannosidase (Harcovics et al., 1994), a rabbit α-1,2-mannosidase (Lal et al., 1994) or a human α-1,2-mannosidase (Tremblay et al., 1998), as well as fungal genes encoding, e.g., an *Aspergillus* α-1,2-mannosidase (Eades et al., 1998), a *Trichoderma reesei* α-1,2-mannosidase (Maras et al., 2000), or a *Saccharomyces cerevisiae* α-1,2-mannosidase (Camirand et al., 1991). Protein sequence analysis has revealed a high degree of conservation among the eukaryotic α-1,2-mannosidases identified so far.

Preferably, the nucleotide sequence for introduction into a *Trichoderma* strain encodes a fungal α-1,2-mannosidase, more preferably, a *Trichoderma reesei* α-1,2-mannosidase, and more particularly, the *Trichoderma reesei* α-1,2-mannosidase described by Maras et al., since it is known to also have a broad substrate specificity (Maras et al., 2000; Van Petegem et al., 2001).

According to the present invention, the nucleotide sequence can encode a full length α-1,2-mannosidase or a functional part thereof. By “functional part” is meant a polypeptide fragment of an α-1,2-mannosidase which substantially retains the enzymatic activity of the full-length protein. By “substantially” is meant that at least about 40%, or preferably, at least 50% or more of the full-length α-1,2-mannosidase activity is retained. Those skilled in the art can readily identify and make functional parts of an α-1,2-mannosidase using a combination of techniques known in the art. Predictions of the portions of an α-1,2-mannosidase essential to or sufficient to confer the enzymatic activity can be made based on analysis of the protein sequence. The activity of a portion of an α-1,2-mannosidase of interest, expressed and purified from an appropriate expression system, can be verified using *in vitro* or *in vivo* assays.

In accordance with the present invention, an α-1,2-mannosidase or a functional part thereof expressed in a *Trichoderma* strain preferably localizes at a place in the secretory pathway where Manα9GlcNAc2 (the substrate of α-1,2-mannosidase) is already formed on a glycoprotein, but has not reached the location of the secretion pathway in which resides the phosphomannosyltransferase. Accordingly, the α-1,2-mannosidase or a functional part thereof is engineered to include an ER-retention signal, such that the protein expressed in a *Trichoderma* strain is targeted to the ER and retains therein for function. “An ER retention signal” refers to a peptide sequence,
which directs a protein having such peptide sequence to be transported to and retained in the
ER. Such ER retention sequences are often found in proteins that reside and function in the
ER. Multiple choices of ER retention signals are available to those skilled in the art, e.g., the
first 21 amino acid residues of the S. cerevisiae ER protein MNS1 (Martinot et al., 1998). A
preferred ER retention signal for use in the present invention is peptide HDEL. The HDEL
sequence found at the C-terminus of a number of yeast proteins acts as a retention/retrieval
signal for the ER (Pelham, 1988). Proteins with an HDEL sequence are bound by a
membrane-bound receptor (Erd2p) and then enter a retrograde transport pathway to return
from the Golgi apparatus into the ER. According to the present invention, an ER retention
signal can be placed anywhere in the protein sequence of an α-1,2-mannosidase, but
preferably at the C-terminal end of the α-1,2-mannosidase.

The α-1,2-mannosidase for use in the present invention can be further modified, e.g., by
insertion of an epitope tag to which antibodies are available, such as Myc, HA, FLAG and His6
tags, which are well-known in the art. An epitope-tagged α-1,2-mannosidase can be
conveniently monitored for both expression and intracellular localization. An ER retention
signal and an epitope tag can be readily introduced into a protein of interest by inserting
nucleotide sequences coding for such signal or tag into the nucleotide sequence encoding the
protein of interest, using any of the molecular biology techniques known in the art.

According to the present invention, the nucleotide sequence coding for an α-1,2-mannosidase
or a functional part thereof can be placed in an operable linkage to a promoter and a 3' termination sequence.

Promoters appropriate for expression of an α-1,2-mannosidase in a Trichoderma strain can
include both constitutive promoters and inducible promoters. Constitutive promoters include e.g., the Aspergillus nidulans glyceraldehyde-3-phosphate dehydrogenase promoter ("the
gpdA promoter"). Examples of inducible promoters include, e.g., the Trichoderma reesei
cellobiohydrolase I promoter ("the CBH-I promoter")

Transcription termination sequences are sequences 3' to the stop codon of a structural gene
which function to stabilize the mRNA transcription product of the gene to which the sequence
is operably linked, such as sequences which elicit polyadenylation. Examples of such 3'
termination sequences are the Trichoderma reesei cellobiohydrolase I terminator ("the CBH-I
terminator") and the A. nidulans indoleglycerolphosphate synthase terminator ("TrypC
terminator").

The preferred vector carrying an α-1,2-mannosidase expression sequence is called
pFGPDGLAT3-MFManHDEL.

Vectors can be introduced into the cells of a Trichoderma strain using known methods such as
the protoplast technique, described by Penttilä et al., 1987. Other published methods useful for
transformation of the plasmids or linear vectors include electroporation (Goldman et al., 1990),
particle bombardment (Lorito et al., 1993) and an Agrobacterium tumefaciens-mediated strategy (de Groot et al., 1998).

During the transformation procedure, the α-1,2-mannosidase expression sequence is cotransformed with a selection plasmid. By 'selection plasmid' is meant a plasmid carrying a selection marker. By 'selection marker' is meant an expression cassette coding for a specific gene product, which enables us to discriminate between a transformed strain and a non-transformed strain. Transformed Trichoderma clones can be selected by using appropriate techniques including but not limited to culturing auxotrophic cells after transformation in the absence of the biochemical product required (due to the cell's auxotrophy), selection for and detection of a new phenotype, or culturing in the presence of an antibiotic which is toxic to the fungus in the absence of a resistance gene within the transformants. Examples of available selection markers for Trichoderma reesei are the acetamidase expression cassette of the vector p3SR2 (Hynes et al., 1983) (enabling transformed strains to grow on acetamide as a sole nitrogen source), the E. coli hygromycin B phosphotransferase cassette of vector pAN7.1 (Punt et al., 1987) and the Streptomyces hindustanus phleomycin-binding protein expression cassette of vector pAN8.1 (Mattan et al., 1988) (enabling the transformed strains to grow on a certain concentration of hygromycin resp. phleomycin).

A further aspect of the invention is a genetically engineered filamentous fungus, expressing a glucosidase II gene according to the invention, further expressing a recombinant N-acetylglucosaminytransferase I gene (GlcNAc-transferase I or GnTI). Preferably, said GnTI gene is a human gene. Even more preferably, said GnTI gene is fused to a Golgi localization signal, preferably a Golgi localization signal derived from a protein with SEQ ID N° 4, even more preferably a Golgi localization signal comprising SEQ ID N° 5, even more preferably a Golgi localization signal essentially consisting of SEQ ID N° 5, most preferably a Golgi localization signal consisting of SEQ ID N° 5. Preferably, said filamentous fungus is a Trichoderma sp., even more preferably said filamentous fungus is Trichoderma reesei Rut-C30.

A GlcNAc-Transferase I is responsible for the addition of β-1,2-GlcNAc to Man₃GlcNAc₂, and converts this core oligosaccharide on glycoproteins to GlcNAcMan₃GlcNAc₂. The mannosic residues of GlcNAcMan₃GlcNAc₂ can be further trimmed by a mammalian Golgi mannosidase II. The resulting GlcNAcMan₃GlcNAc₂ structure can be further elongated with other glycan residues to form hybrid or complex type sugar branches characteristic of mammalian glycoproteins. Thus, by way of introducing a GlcNAc-transferase I into filamentous fungi such as Trichoderma reesei, glycoproteins with a mammalian-like or cognate glycoprotein pattern can be produced.
According to the present invention, the nucleotide sequence encoding a GlcNAc-transferase I (GnTI) for use in the expression vector of the present invention can derive from any higher eukaryotic species, e.g., rabbit (Sariar et al., 1991; SWISS-PROT Accession No P27115), human (Schachter, 1991; SWISS-PROT Accession No P26572), rat (Fukuda et al., 1994; SWISS-PROT Accession No Q09325), plants and insects. Preferably, the nucleotide sequence for use in the present vectors encodes a human GnTI. More preferably, the GnTI gene comprises SEQ ID N° 1, even more preferably, the GnTI gene is essentially consisting of SEQ ID N° 1, most preferably, the GnTI gene is consisting of SEQ ID N° 1.

According to the present invention, the nucleotide sequence can also encode only a functional part of a GlcNAc-Transferase I. By "functional part" is meant a polypeptide fragment of a GlcNAc-Transferase I, which substantially retains the enzymatic activity of the full-length protein. By "substantially" is meant at least about 40%, or preferably, at least 50% or more of the enzymatic activity of the full-length GlcNAc-Transferase I is retained. For example, as illustrated by the present invention, the catalytic domain of the human GnTI constitutes a "functional part" of the human GnTI. Those skilled in the art can readily identify and make functional parts of a GlcNAc-Transferase I using a combination of techniques known in the art. Predictions of the portions of a GlcNAc-Transferase I essential to, or sufficient to confer the enzymatic activity can be made based on analysis of the protein sequence. The activity of a portion of a GlcNAc-Transferase I of interest, expressed and purified from an appropriate expression system, can be verified using in vitro or in vivo assays.

In accordance with the present invention, a GnTI or a functional part thereof expressed in a *Trichoderma reesei* strain preferably is targeted to a site in the secretory pathway where Man$_n$GlcNAc$_2$ (the substrate of GnTI) is already formed on a glycoprotein. Preferably, the GnTI or a functional part is targeted to the Golgi apparatus.

Accordingly, in a preferred embodiment of the present invention, the GnTI is engineered as such that the GnTI or a functional part thereof expressed from the vector is fused with a fungal Golgi localization signal. "A fungal Golgi localization signal" refers to a peptide sequence, which directs a protein having such a peptide sequence to be retained in the Golgi apparatus. Such Golgi localization sequences are often found in proteins that reside and function in the Golgi apparatus. Choices of Golgi localization signals are available to those skilled in the art. A preferred Golgi localization signal for use in the present invention is a peptide derived from the N-terminal part of a *Saccharomyces cerevisiae* Kre2 protein (ScKre2). According to the present invention, a Golgi localization signal can be placed anywhere within the GnTI, but preferably at the terminus of the GnTI, and more preferably at the N-terminus of the GnTI.

The GnTI for use in the present invention can be further modified, e.g., by insertion of an epitope tag to which antibodies are available, such as Myc, HA, FLAG and His6 tags well known in the art. An epitope-tagged GnTI can be conveniently purified, or monitored for both
expression and intracellular localization. A Golgi localization signal and an epitope tag can be readily introduced into a protein of interest by inserting nucleotide sequences coding for such signal or tag into the nucleotide sequence encoding the protein of interest, using any of the molecular biology techniques known in the art.

According to the present invention, the nucleotide sequence coding for a GlcNAc transferase I or a functional part thereof can be placed in an operable linkage to a promoter and a 3' termination sequence.

Promoters appropriate for expression of a GlcNAc transferase I in a Trichoderma strain can include both constitutive promoters and inducible promoters. Constitutive promoters include e.g., the Aspergillus niger glyceraldehyde-3-phosphate dehydrogenase promoter ("the gpdA promoter"). Examples of inducible promoters include, e.g., the Trichoderma reesei cellobiohydrolase I promoter ("the CBHI promoter").

3' termination sequences are sequences 3' to the stop codon of a structural gene which function to stabilize the mRNA transcription product of the gene to which the sequence is operably linked, such as sequences which elicit polyadenylation. Examples of such 3' termination sequences are the Trichoderma reesei cellobiohydrolase I terminator ("the CBHI terminator") and the A. nidulans indoleglycerolphosphate synthase terminator ("TrypC terminator").

The preferred vector carrying a GlcNAc transferase I expression sequence is called pFGPDKrecohGnTl.

Vectors can be introduced into the cells of a Trichoderma strain using known methods such as the protoplast technique, described by Penttila et al., 1987. Other published methods useful for transformation of the plasmids or linear vectors include electroporation (Goldman et al., 1990), particle bombardment (Lorito et al., 1993) and an Agrobacterium tumefaciens-mediated strategy (de Groot et al., 1998).

During the transformation procedure, the GlcNAc transferase I expression sequence is cotransformed with a selection plasmid. By 'selection plasmid' is meant a plasmid carrying a selection marker. By 'selection marker' is meant an expression cassette coding for a specific gene product, which enables us to discriminate between a transformed strain and a non-transformed strain. Transformed Trichoderma clones can be selected by using appropriate techniques including but not limited to culturing auxotrophic cells after transformation in the absence of the biochemical product required (due to the cell's auxotrophy), selection for and detection of a new phenotype, or culturing in the presence of an antibiotic which is toxic to the fungus in the absence of a resistance gene within the transformants. Examples of available selection markers for Trichoderma reesei are the ampicillin resistance cassette of the vector pC102 (Hynes et al., 1983) (enabling transformed strains to grow on ampicillin as a sole nitrogen source), the E. coli hygromycin B phosphotransferase cassette of vector pAN7.1
(Punt et al., 1987) and the *Streptococctileichus hindustanus* phleomycin-binding protein expression cassette of vector pAM8.1 (Matern et al., 1988) (enabling the transformed strains to grow on a certain concentration of hygromycin resp. phleomycin).

Another aspect of the invention is a filamentous fungus expressing a recombinant glucosidase II gene, according to the invention, further expressing both a recombinant α-1,2-mannosidase gene and a recombinant GlcNAc-transferase I gene.

Still another aspect of the invention is the use of a genetically modified filamentous fungus, according to the invention, to modulate protein secretion, compared with the parental strain.

**Brief description of the figures**

**Figure 1:** Schematic overview of the inverse PCR strategy.

**Figure 2:** Construction strategy for the glucosidase II expression plasmids pFGPDgsILvreesei and pFGPDgsILvreeseiMyc.

**Figure 3:** (A) PCR on Rut-C30 cDNA library using degenerate primers 1 and 3. (B) nested PCR on Rut-C30 cDNA library using degenerate primers 1, 2 and 3.

**Figure 4:** PCR screening with degenerate primers 1 and 3: (A) first round with about 5000 clones per well; (B) second round with about 500 clones per well; (C) third round with about 50 clones per well. The cell suspension from well A2 was used for the second PCR round; the cell suspension from well F3 was used for the third PCR round and the cell suspension from well B9 was used for colony hybridization analysis.

**Figure 5:** Results of the colony hybridization.

**Figure 6:** Plasmid DNA of the 7 positive clones was prepared and digested with XhoI/EcoRI to isolate the cDNA insert. Hybridization analysis indicates that at least the 1700bp fragment is glucosidase II specific.

**Figure 7:** (A) cloning of the 5’ part of the gll ORF by inverse PCR; (B) cloning of the 5’ part of the gll ORF by RACE; (C) sequence comparison between the inverse PCR and the 5’ RACE fragment reveals the existence of an intron region.

**Figure 8:** Glycosylation profile of the RutC30, QM9414 and g14 transformants, either native, after α-1,2-mannosidase digestion or after mild acid hydrolysis. For all three cases it is clear that the g14 transformant has a glycosylation profile that contains characteristics of both the RutC30 and the QM9414 strains. The deduced N-glycans are numbered: 1: Man5GlcNAc2; 2: Man5GlcNAc2; 3: Man5GlcNAc2; 4: Man5GlcNAc2; 5: GlcMan5GlcNAc2; 6: GlcMan5GlcNAc2; 7: GlcMan5GlcNAc2; 3’: ManPMan5GlcNAc2; 4’: ManPMan5GlcNAc2; 5’: ManPMan5GlcNAc2; 5’: PMan5GlcNAc2.

**Figure 9:** Southern blot analysis of the genomic DNA of several hygromycin resistant transformants and of the WT RutC30 strain: transformant g14 expresses both the mutant and the repaired glucosidase II alpha-subunit gene; all other transformants grow on hygromycin but
have not integrated the repaired glucosidase II alpha-subunit gene into the genome. The fragment of 3400 bp (arrow) indicates the random integration of the glucosidase II expression cassette. The fragment of 5000 bp represents the hybridisation signal against the endogenous mutant glucosidase II alpha subunit gene. Ref: PstI digested lambda DNA as reference.

**Figure 10:** Construction strategy for the α-1,2-mannosidase expression plasmid pFGPDLAT3-MFMenHDEL.

**Figure 11:** Construction strategy for the GlcNAc transferase I expression plasmid pFGPDKrecoGnTl.

**Figure 12:** N-glycan analysis of several transformants capable of growing on acetamide as single N-source: transformants F4, F17, F18 and F32 almost exclusively synthesize Man9GlcNAc2 as a result of the expression of an ER-localized α-1,2-mannosidase.

**Figure 13:** PCR analysis of the genomic DNA of a few acetamide resistant *Trichoderma* clones: Transformants F4, F17, F18 and F32 score positive for the PCR analysis.

**Figure 14:** Probability of coiled coil structure as predicted by the paircoil algorithm.

A: predicted coiled coil of GnTl, the maximal probability is 0.36. B: predicted coiled coil of yeast Kre2, maximal probability 0.69.

**Figure 15:** Each gel represents separate experiments in which the secretion level of the g14 transformant and the RutC30 wild type strain were compared with one another. For each analysis, the different protein samples were prepared from different but simultaneously grown cultures of both strains. In the first gel, Hygr1 and Hygr2 represent hygromycin resistant RutC30 transformants that have no genomic integration of the full-size glucosidase II (checked on gDNA and via N-glycan analysis). As a result, they have a similar secretion behaviour as the untransformed RutC30 strain.

**Figure 16:** Strategy for the construction of a S. cerevisiae rot2 knock out, and for the consequent replacement of the URA3 cassette by a mutant glucosidase II gene, carrying the Rut C30 *T. reesei* glucosidase II mutation

**Figure 17:** DSA-FACE analysis of the rot2 knock out transformants (KO16, KO18, KO20) as confirmed by PCR, in comparison with a transformant with an aberrant PCR pattern (KO11) and the parental strain YA-72, and with the rot2 knock out mutant Y13369 and its parental strain BY4742. All rot2 knock outs show a similar sugar pattern that is clearly different from that of the wild type strains.

**Figure 18:** IFNβ-specific Western blot of proteins secreted in the medium by 8 BY4742 IFNβ producing clones (1-8) and 8 Y13369 IFNβ producing clones (A-H). M: marker; WT: non-transformed BY4742 parental strain; KO: non-transformed Y13369 rot2 knock out mutant. The average OD<sub>600</sub> value of the cultures was 12.56 for the BY4742 transformants and 12.65 for the Y13369 transformants. The upper band is the glycosylated form, the lower band is the not glycosylated form.
Figure 19: IFNβ- specific Western blot of pooled medium proteins from cultures of 8 BY4742 IFNβ producing clones (WT), 8 Y13369 IFNβ producing clones (KO) and 8 Y13369 IFNβ producing clones supertransformed with pYX132LEUGLS1mut3' (mut). M: marker, C1 and C2: untransformed parental strains. The upper band is the glycosylated form, the lower band is the not glycosylated form.

Examples

Materials and methods

Strains and transformation procedure:

Two *Trichoderma reesei* strains were used for the glucosidase II work, being the Rut-C30 (ATCC 56765) and the QM9414 (ATCC 26921) strain. *Trichoderma* transformations were by co-transformation according to Penttila et al. (1987) using the hygromycin resistance gene (plasmid pAN7.1 (Punt et al., 1987)) as a selection marker. Before transformation, the glucosidase II expression vectors pFGPDglsITreesei and pFGPDglsITreeseiMyc were linearized with *FspI* (Biolabs). Transformants were selected on minimal medium (composition per liter: 20 g dextrose monohydrate, 5 g (NH₄)₂SO₄, 15 g KH₂PO₄, 0.3 g CaCl₂, 0.3 g MgSO₄ and mineral components) containing 150 μg/ml of hygromycin.

*Trichoderma reesei* QM9414 was used for the expression of an ER-localized α,1,2-mannosidase. Transformation was by co-transformation according to Penttila et al. (1987) using *AmdS* (plasmid pSSR2, (Hynes et al., 1983)) as a selection marker. Before transformation, the α,1,2-mannosidase expression plasmid was linearized with *NdeI* (Biolabs). Transformants were selected on minimal medium with acetamidase as the sole nitrogen source (composition per liter: 20 g dextrose monohydrate, 15 g KH₂PO₄, 0.3 g CaCl₂, 0.3 g MgSO₄, mineral components, 10 ml 1M acetamidase and 12.5 ml 1M CsCl).

*Trichoderma reesei* QM9414-F4 was used for the expression of a Golgi-localized GlcNAC-transferase I. This strain is a functional α,1,2-mannosidase transformant of strain QM9414. Transformation was by co-transformation according to Penttila et al. (1987) using the phleomycin resistance gene as a selection marker. Before transformation, the GlcNAC-transferase I expression plasmid was linearized with *NdeI* (Biolabs). Transformants were selected on minimal medium (composition per liter: 20 g dextrose monohydrate, 15 g KH₂PO₄, 5 g (NH₄)₂SO₄, 0.3 g CaCl₂, 0.3 g MgSO₄, mineral components) containing 150 μg/ml zeocin (Invitrogen).

For the cloning of the glucosidase II gene and for the construction work, we used electrocompetent resp. chemocompetent *E. coli* MC1061 cells (hsdR2 hsdM' hsdS' araD139 Δ[ara leu]lacZYΔΔlac-pro galE15 galK16 rpsL (Str) mcrA mcrB1) (Casadaban et al., 1980). Growth and transformations were as described in Sambrook et al., (1989).
Saccharomyces cerevisiae YA-72 (MATa, his3, ura3, leu2) is an IFN-β producing yeast strain, obtain by transforming the strain CL3-ABYS88 with a GAL1-MF(5)-IFNβ-CYCT integrative expression cassette (Demolder et al., 1994). S. cerevisiae Y13369 is a rct2 knock out (MATa, his3, leu2, ura3, YBR229c::kanMX4) from BY4742 and was obtained from EUROFAN. The parental strain BY4742 was used as reference.

Yeast strains were transformed using the LiAc method.

Nucleic acid preparations from filamentous fungi

Trichoderma genomic DNA was prepared from 5 to 6 day old mycelium, grown in shake flasks in minimal medium (composition per liter: 20 g dextrose monohydrate, 5 g (NH₄)₂SO₄, 15 g KH₂PO₄, 0.3 g CaCl₂, 0.3 g MgSO₄ and mineral components) at 30°C. The mycelium was separated from the growth medium and ground using liquid nitrogen. 5 to 10 ml of extraction buffer (200 mM Tris HCl pH 8.5; 250 mM NaCl; 0.5% SDS) was added to resuspend the disrupted Trichoderma cells. An equal amount of a phenol/chloroform/isoamyl alcohol mixture (25/24/1) was added to the suspension. After mixing, samples were centrifuged for 1 hour at 2500g. The upper phase, containing the DNA, was transferred to a new tube and incubated with 1 mg of RNaseA for 30 minutes at 37°C. Following a new extraction with an equal volume of a mixture of chloroform/isoamyl alcohol (24/1), the upper phase was transferred to a new tube. The DNA was precipitated with half a volume of isopropanol (centrifugation at full speed for 20 min. at 4°C). After removing the supernatant, the DNA pellet was washed with 70% EtOH, dried at 37°C and resuspended in a suitable volume of H₂O.

Total Trichoderma RNA was prepared from 5 to 6 day old mycelium, grown in shake flasks in minimal medium at 30°C. The mycelium was separated from the growth medium and ground using liquid nitrogen. Per 0.2 g of mycelium, 1 ml of extraction buffer (25 mM sodium citrate; 4 M GuHCl; 100 mM sodium-lauryl sarcosine and 100 mM beta-mercapto-ethanol) was added. The suspension was thoroughly mixed and incubated at 50°C for 15 minutes. An equal amount of a phenol/chloroform/isoamyl alcohol mixture (25/24/1) was added to the suspension. After mixing, samples were centrifuged for 15 minutes at 9000g and 4°C. This extraction was repeated twice and followed by a chloroform/isoamyl alcohol (24/1) extraction. After centrifugation (4°C, 9000g, 15 minutes), the upper phase was collected. One volume of 6 M LiCl, was added and the RNA was precipitated overnight at 4°C. After centrifugation (4°C, 9000g, 15 minutes), the obtained RNA pellet was resuspended in one volume of 3 M LiCl and again precipitated through centrifugation (4°C, 9000g, 15 minutes). The pellet was resuspended in 400 µl of 0.3 M NaOAc pH 5.7 and incubated at 50°C for 10 minutes. After centrifugation (4°C, 9000g, 15 minutes), the supernatant was collected. 1 ml of ice-cold EtOH was added and the RNA was precipitated overnight at -20°C. The suspension was centrifuged
at 4°C for 20 minutes and the obtained pellet was washed with 70% EtOH. The dried pellet was resuspended in a suitable volume of DEPC treated H₂O.

Cloning of the *Trichoderma* glucosidase II gene

Cloning of the glucosidase II alpha subunit was initiated from a *Trichoderma reesei* Rut-C30 cDNA library (Marja Penttilä, VTT Biotechnology). In this library, which contains about 100,000 clones, the Rut-C30 cDNA was cloned into an EcoRI/Xhol opened pAJ401 yeast expression vector (Saloheimo et al., 1994). Based on the alignment of several known mammalian and yeast alpha subunit amino acid sequences, three homologous regions were selected on which degenerate primers were synthesized: the sense primer 5’-GTTATGGHCCIGAGCATGC-3’ (= primer 1) and the antisense primers 5’-GIGCGTGIICCIKGGAAGAIG-3’ (= primer 2) and 5’-TGISWICCIGGAAGAAIGGIC-3’ (= primer 3), with H = A, C and T; K = G and T; S = G and C; W = A and T. Amplification with primers 1/3 and with primers 1/2 should result in a DNA fragment of approximately 1170 bp. Reaction conditions for the amplification with primers 1/3 were the following: 94°C for 45 sec.; 59°C for 1 min., 72°C for 1.5 min. Similar reaction conditions were used for the nested PCR, except for the annealing temperature which was decreased to 50°C. Obtained PCR fragments were cloned into pCR2.1-TOPO (Invitrogen) for sequence analysis. TOPO-cloning was done as described by the manufacturer.

As a screening strategy for a bacterial clone containing the *T. reesei* glucosidase II alpha subunit, we used the technique of ‘Rapid cDNA cloning by PCR screening’ (Takumi and Lodish, 1994). In brief, the cDNA library was transformed to *E. coli* MC1061 competent cells. The transformation mixture was diluted and divided into a 96 well plate in a way that every well contained about 5000 cDNA clones. As such, the whole microtiter plate represented about 5 times the number of cDNA clones within the library. After incubation for several hours at 37°C, a PCR was performed with primers 1 and 3 as described above on cellular mixtures of the 12 columns and the 8 rows of the 96 well plate. Based on these results positive wells, lying on the crossing of positive columns and positive rows, could be identified. The cell suspension of one of the positive wells was inoculated into the wells of a new microtiter plate at 500 clones per well. The PCR strategy was repeated and the cell suspension of one of the resulting positive wells was again inoculated into the wells of a new microtiter plate, this time at a concentration of 50 clones per well. By using the PCR strategy, again new positive wells were identified. From one of these wells, the cell suspension was plated on solid Luria Bertani medium. About 200 colonies were transferred to Hybond N filters (Amersham), incubated overnight and analyzed through colony hybridization using the *Trichoderma* glucosidase II specific 1170 bp PCR fragment as probe. ³²P-labeling of the probe was done using the High-Prime kit (Roche), following the instructions of the manufacturer.
DNA was prepared from several positive clones and digested with EcoRI (Gibco BRL) and XhoI (Gibco BRL) to release the cDNA insert. The glucosidase II specificity of the obtained fragments was checked by Southern blotting, using the 32P-labeled 1170 bp PCR fragment as probe. Also, the obtained fragments were cloned for sequence analysis either as an EcoRI/XhoI fragment into an EcoRI/SalI (Roche) opened pUC19 vector or as a blunt XhoI fragment into an EcoRV (Gibco BRL) opened pBluescript II KS +/- (Stratagene) vector.

5'-RACE and inverse PCR

To clone the 5' missing part of the glucosidase II alpha subunit gene, both 5'-RACE and inverse PCR were used. For the inverse PCR (iPCR) strategy, an antisense (ROT2TR4_AS: 5'-GTAAACGTTCGTTCCCACC-3') and sense (ROT2TR1_S: 5'-GGCTCCATCCCTTTCTCATGC-3') PCR primer were designed, based on the 5' sequence of the cloned but incomplete glucosidase II alpha subunit Rut-C30 cDNA. The 5' end of the primers is facing each other and hybridizes to positions on the cDNA that are separated by 229 bp containing an Ncol restriction site. 10 μg of genomic Trichoderma DNA was digested at 37°C for several hours with 100 units BamHI (Gibco BRL), a restriction enzyme that cuts the cloned cDNA sequence, 3' to both iPCR primers. After heat inactivation of BamHI (10 minutes at 65°C), the obtained genomic DNA fragments were induced to self-circulate through overnight incubation at room temperature in the presence of 5 units T4 DNA ligase (Roche). Following a phenol extraction and isopropanol precipitation, the DNA was digested with 80 units Ncol (Biolabs) for several hours at 37°C. As such, the desired glucosidase II containing genomic DNA fragment will be linearized again, enabling the designed iPCR primers (now facing each other with their 3'ends) to hybridize to each one end of the fragment. Following a new phenol extraction and isopropanol precipitation, the DNA was resuspended into 50 μl of H2O. 1 μl of this DNA suspension was used as a template in a PCR reaction with 50 pmol of each iPCR primer. The PCR reaction was performed with cloned Pfu polymerase (Stratagene) in a total volume of 100 μl, and consisted of 20 cycles of 94°C for 45 sec.; 55°C for 30 sec. and 72°C for 1 min. 30 sec. A schematic overview of the inverse PCR strategy is shown in figure 1.

For the 5'-RACE procedure, we made use of the First Choice™ RLM-RACE strategy kit from Ambion. Primer design and experimental procedure was done on total RNA, following the instructions of the manufacturer. For the outer PCR primer ROT2TR-RLMRACE (5'-GATATACGAGACGTCGG-3') was used. For the inner PCR, we used primer ROT2TR4_AS (5'-GTAAACGTTCGTTCCCACC-3'). Annealing during the outer PCR reaction was performed at 57°C; for the inner PCR, a temperature of 55°C was used.
The 5'-RACE and inverse PCR fragments were cloned into the pCR-blunt II-TOPO vector (Invitrogen) for sequence analysis, following the instructions of the manufacturer.

**Intron and frame-shift analysis through PCR**

The intron-exon composition of the glucosidase II gene was analyzed by amplifying the whole gene from the Rut-C30 genome. 1 μg of gDNA was used as template; the sequence of the sense resp. antisense primer was 5'-ATGAGGTCCACGTAGGGG-3' resp. 5'-AGCCAGCTTGATGCTCC-3'. Using **Pfu** polymerase (Stratagene), following reaction conditions were applied: 25 cycles of 94°C for 1 min.; 55°C for 1 min. and 72°C for 7 min.

Frame-shift analysis was done by PCR on the Rut-C30 and QM9414 genomes. 1 μg of gDNA was used as PCR template. The sequence of the internal glucosidase II specific primers was 5'-TATCTCTGTTTCCGGTCTCG-3' for the sense primer ROT2TR3_S and 5'-CTGGTCATCAATCGCCAAGCC-3' for the antisense primer ROT2TR0_AS. PCR was performed using **Pfu** polymerase and following reaction conditions: 25 cycles of 94°C for 1 min.; 60°C for 1 min. and 72°C for 1 min.

The PCR fragments were cloned into the pCR-blunt II-TOPO vector (Invitrogen) for sequence analysis, following the instructions of the manufacturer.

**Construction of the Trichoderma expression vector for a functional Trichoderma glucosidase II alpha subunit gene**

In a first step, the cloned glucosidase II cDNA fragment was cut out of the pAJ401 library vector as an approximately 3000 bp **EcoRI/HindIII** (GibcoBRL) fragment. This fragment was ligated into an **EcoRI/HindIII** opened pUC19 vector, resulting in plasmid pUC19AgslITrees(e(shift). In a second step, the frame-shift within the cloned Rut-C30 cDNA fragment was repaired. Using genomic DNA from the QM9414 strain as a template and **Pfu** polymerase (Stratagene), a PCR reaction was started with primers ROT2TR2_S (5'-ATCAAATGACCAACTCTCGGC-3') and ROT2TR0_AS (5'-CTGGTCATCAATCGCCAAGCC-3'). The PCR reaction went on for 25 cycles of 1 min. at 95°C, 1 min. at 60°C and 1 min. at 72°C. The obtained fragment was digested with **XcmI** (Biolabs)/**PstI** (Biolabs) and ligated into the pUC19AgslITrees(e(shift), resulting into the vector pUC19AgslITrees(e(repaired). In a third step, the ORF of the glucosidase II alpha subunit was completed: for this the 5' RACE fragment (materials and methods 4) was digested with ** DraIII** (Biolabs) and **MspI** (Biolabs) and ligated into the **DraIII/EcoRI**-Klenow (Roche) treated vector pUC19AgslITrees(e(repaired), resulting into the plasmid pUC19AgslITrees. In a next step, a unique **SmaI** site was incorporated at the 3' terminus of the glucosidase II ORF through mutagenesis, using the Quick Change Mutagenesis kit form Stratagene. The primer couple used to induce the silent mutation (from CGT to CGG) consisted of a sense primer...
5'-CCATGTGAAGGCCGGGTGGGATGACTGC-3' and an antisense primer 5'-CCAGTCATCCCCGAGCCCGTACATGGG-3'. The resulting plasmid was called pUC19GlsilTreesei(SmaI). In a following step, the plasmid was cut EcoRI/SalI for the integration of a linker at the 5' end of the glucosidase II ORF. The linker consisted of two partially complementary primers (sense primer: 5'-GAATTCCCGGCGGTAGCAAATTATGAGG-3' and antisense primer: 5'GTGACCGCTCTACATATTACGTACCAGGGGG-3') and was prepared by mixing both primers, boiling the mixture and gradually cooling it to room temperature. By inserting the linker, two new and unique restriction sites (SacII and SmaI) were integrated at the 5' end of the glucosidase II ORF, creating plasmid pUC19(5'glsilTreesei(SmaI)). In a next step, this plasmid was opened HindIII/SacII-T4 (Roche) treated and ligated into the HindIII/NcoI (Boehringer)-S1 (Gibco BRL) treated plasmid pFGPDGLAT3 (Contreras et al., 1991). As such the glucosidase II alpha subunit ORF was placed under the transcriptional control of the constitutive \textit{A. nidulans} gpdA promoter. To decrease the distance between the 3' end of the ORF and the TrpC terminator, the vector was digested with MluI (Gibco BRL) to remove a fragment of about 500 bp. The obtained vector fragment was closed by overnight ligation, resulting in the plasmid pFGPDglsilTreesei. A variant of this plasmid was constructed, containing the \textit{Trichoderma} glucosidase II ORF with a C-terminal Myc-tag. For this, vector pUC19(5'glsilTreesei(SmaI)) was digested with Smal (Gibco BRL) and SmaI (Boehringer). The resulting fragment containing most of the glucosidase II ORF, was ligated into an NcoI (S1 treated)/BspI120I (MBL Fermentas) (Klenow treated) opened pFGPDglScMyC vector. Using this construction strategy the 10 C-terminal amino acids of the \textit{Trichoderma} glucosidase II were replaced by the coding sequence for the Myc-tag. In the resulting vector, called pFGPDglsilTreeseiMyc, the ORF coding for the Myc-tagged \textit{Trichoderma} glucosidase II alpha subunit is under the transcriptional control of the constitutive \textit{A. nidulans} gpdA promoter and the TrpC terminator. Plasmid pFGPDglsilScMyc was constructed for the expression of the S. cerevisiae glucosidase II alpha subunit in \textit{Trichoderma reesei}. This vector was constructed as follows: by a PCR strategy using plasmid pGAPZglsilScMyc as DNA template, \textit{Pfu} polymerase, sense primer R0T2ScNco_S 5'-CTTGGCCATGCTCCTTTAGAATGGCTC-3' and antisense primer R0T2ScMycHind_A5 5'-CCAAGCGCTCAGATCCCTCTGAGATGAG-3', we amplified a Myc-tagged version of the \textit{S. cerevisiae} glucosidase II gene. The PCR reaction consisted of 30 cycles of 45 sec. at 94°C, 5 sec. at 50°C and 8 min. at 72°C. Since the nucleotide sequences of the NcoI and HindIII restriction sites were incorporated in the sense resp. antisense primer, the obtained PCR fragment was easily cloned into an NcoI/HindIII opened pFGPDGLAT2 vector, resulting into plasmid pFGPDglsilScMyc. Vector pGAPZglsilScMyc was constructed for the expression of the \textit{S. cerevisiae} glucosidase II ORF in \textit{Pichia pastoris} (WO20020856). Genomic DNA was prepared form the \textit{S. cerevisiae} strain InvSC1(α, leu2-3, 112 his3a1, trpl-289, ura3-52) (Invitrogen) using the Nucleon kit.
(Amersham). This was used as template for the amplification of the glucosidase II alpha subunit with sense primer ROT2Sc_S 5'-CCGCTCGAGATGTCTTTAGAATGACTC-3' (containing the sequence for a unique XhoI restriction site) and antisense primer ROT2Sc_AS 5'-CCGGGCCAAAATAACTCTCATCCTGA-3' (containing the sequence for a unique Apal restriction site). Amplification was performed by a touch-down PCR strategy using LA TaKaRa polymerase (TaKaRa Shuzo co., LTD.) with the following conditions: 3 cycles of 30 sec. at 94°C, 2 sec. at 98°C, 30 sec. at 65°C and 10 min. at 70°C; 3 cycles of 30 sec. at 94°C, 2 sec. at 98°C, 30 sec. at 60°C and 10 min. at 70°C; and 30 cycles of 30 sec. at 94°C, 2 sec. at 98°C, 30 sec. at 55°C and 10 min. at 70°C. After digestion with Apal (Biolabs)/XhoI (Gibco BRL), the fragment was ligated into an Apal/XhoI opened pGAPZ.A vector (Invitrogen), to allow in-frame cloning of the amplified glucosidase II ORF with a nucleotide sequence coding for the Myc-tag. The resulting plasmid was called pGAPZglsIfMyc. An overview of the construction strategy can be seen in figure 2.

Construction of the α-1,2-mannosidase and GloNAc-transferase expression plasmids

For the expression of the ER-localized variant of the Trichoderma reesei α-1,2-mannosidase in 5 Trichoderma reesei Rut-C30-g31, the α-1,2-mannosidase coding part was isolated from plasmid pGAPZMFManHDEL. This plasmid contains the mannosidase with the N-terminal prepro-signal sequence of the S. cerevisiae α-mating factor and a C-terminal HDEL-tag as described in Callewaert et al. (2001b). The mannosidase part was isolated by a BamHI (Biolabs)/NotI (Biolabs) digest. The BamHI sticky end was blunted with T4-polymerase (Roche). The obtained fragment was ligated in an NcoI (Biolabs) (Mung Bean nuclease (Roche) blunted)/NotI opened pFGPDGLAT3 (Contreras et al., 1991) vector. The resulting plasmid pFGPDGLAT3-MFManHDEL contains the α-1,2-mannosidase ORF under the transcriptional control of a constitutive gpdA promoter. An overview of the construction scheme is presented in figure 11.

In order to target more efficiently the human GloNAc-transferase I to the fungal Golgi apparatus, the GnTII N-terminal part was replaced by the S. cerevisiae Kre2 N-terminal sequence, known to be responsible for protein retention in the yeast Golgi (Lussier et al., 1995). Plasmid pFGPDKrecoGnTI was constructed as follows. Plasmid YEp352Kre2 (kindly provided by Dr. Howard Bussey, McGill University, Montreal, Canada), which contains the Kre2 gene as a SacI/PvuII fragment cloned in a Sall(Klenow blunted)/SacI opened YEp352 vector, was digested with SacI (Biolabs)/PvuII (Gibco BRL) and T4-polymerase (Roche) blunted. The 5'end region of the gene was isolated and cloned in a Klenow blunted SgrAl (Roche)/XbaI (Gibco BRL) opened pUCHGnTI vector (Maras et al., 1997). By doing so, the coding sequence of the Golgi localization signal of the yeast Kre2 protein was cloned in frame with the nucleotide sequence of the catalytic domain of the GloNAc transferase I protein. The
resulting ORF was isolated by performing an EcoRV (Gibco BRL)/HindIII (Gibco BRL) double digest and was cloned into an NcoI (S1-nuclease (Gibco BRL) blunted)/HindIII opened pFGPDGLAT3 vector, as such creating the plasmid pFGPDxKrecoGnTl. The construction of the expression plasmid is presented in figure 11.

Genomic analysis

For the analysis of the glucosidase II transformants, genomic DNA was digested overnight with 50 units of NheI (Biolabs) and KpnI. After electrophoresis, the DNA was transferred to a Hybond N* membrane (Amersham). Integration of the expression plasmid into the genome was checked, by hybridizing the Hybond filter with a 32P-labeled glucosidase II-specific probe. Labeling of the probe was done using the High Prime kit (Roche). The DNA template for the labeling reaction consisted of a part of the glucosidase II ORF and was obtained through an NcoI digest on plasmid pFGPDglsIITreseei.

A similar strategy was followed after digestion of the genomic DNA with 50 u of DraI and BglII (Biolabs). This time however, the Southern blot was screened with a probe which is derived from an EcoRI/NheI fragment of vector pFGPDglsIITreseei and which hybridizes against the gpdA promoter sequence of the glucosidase II expression plasmid.

For the analysis of the α-1,2-mannosidase transformants, genomic DNA was digested overnight with 50 units BglII (Promega) and NcoI (Promega). After electrophoresis, the DNA was transferred to a Hybond N* membrane (Amersham). Integration of the expression plasmid into the genome was checked by hybridizing the Hybond filter with a 32P-labeled α-1,2-mannosidase-specific probe. Labeling of the probe was done using the High Prime kit (Roche). The DNA template for the labeling reaction consisted of a part of the gpdA promoter and was obtained through an EcoRI (Promega)/NheI (Biolabs) digest on plasmid pFGPDGLAT3-ManHDEL. Integration was also checked by PCR on 1 μg gDNA using Taq polymerase (MBI Fermentas). A gene-specific antisense primer hybridizing against the 3' region of the mannosidase gene (5'-CAACTCGTCTGAGCAAGG-3'), and a sense primer that hybridizes against the gpdA promoter region of the expression vector (5'-CCATATTTTCTGGTCTCCC-3'), were used for the amplification reaction. The PCR conditions were as follows: 30 cycles of 1 min. at 94°C, 1 min. at 60°C and 2 min. at 72°C.

For the analysis of the GlcNAc-transferase I transformants, genomic DNA was digested overnight with 50 units BglII (Promega). After electrophoresis, the DNA was transferred to a Hybond N* membrane (Amersham). Integration of the expression plasmid into the genome was checked, by hybridizing the Hybond filter with a 32P-labeled GlcNAc transferase I-specific probe. Labeling of the probe was done using the High Prime kit (Roche). The DNA template for the labeling reaction consisted of a part of the GlcNAc transferase I ORF and was obtained through a BglII/NcoI digest on plasmid pFGPDxKrecoGnTl.
Construction of the S. cerevisiae plasmids

pSCGALMFHIFNB2 is an IFNβ expression construct where the IFNβ coding sequence is placed under control of the GAL promoter (Demolder et al., 1994)

The 3' end of the ROT2 gene was isolated by PCR reaction using 5'TACGGGCGGGGAAAAAACGAAATGATATC3' as sense primer and 5'CTTTGCTGAGGTTGGAATGTCC3' as antisense primer. The PCR conditions used were 95°C for 3 min; 94°C for 1 min; 55°C for 1 min; 72°C for 1 min; 25 cycles; 72°C for 10 min; cool down to 4°C. The resulting fragment was cloned into pCR2.1-TOPO (Invitrogen Co, Carlsbad, CA, USA) to yield pCR2.1-TOPO3ROT2.

pGAPADE1glsII was constructed as follows: the glucosidase II ORF of S. cerevisiae was amplified from the gDNA of strain INVSc (α leu2-3, 112 his3Δ1, trp1-289, ura3-52) (Invitrogen). gDNA was prepared from an overnight grown yeast culture in YPD at 30°C. DNA was prepared using the Nucleon Kit for extraction of yeast gDNA (Amersham). The sense primer for the PCR amplification hybridizes to the 5' part of the yeast ORF (including the ATG start coding) and contains a Xhol restriction site for easier downstream cloning work. The antisense primer hybridizes against the 3' part of the ORF (but not including the stop codon) and contains an Apal site for easier downstream cloning. The sequence of both primers is as follows: sense primer ROT2(S): 5'-CCGCTCGAGATGGTCCCTTTTGAATGGCTC-3' and antisense primer ROT2(AS): 5'-CGGCGCGGAAAAATACCTTCCAATCTTCAG-3'.

PCR was done via a touch-down strategy using LA TaKaRa (ImTec Diagnostics) on 200 ng gDNA, using 50 pmol of each primer. The amplification was obtained during 3 rounds of 94°C for 30 sec. – 98°C for 2 sec. – 65°C for 30 sec. – 70°C for 10 min., followed by 3 similar PCR rounds, however this time with an annealing temperature of 60°C, followed by 30 similar PCR rounds, however this time using an annealing temperature of 55°C.

A fragment of the expected length of 2900 bp was obtained via this PCR strategy and was XhoI/ApaI ligated into a XhoI/ApaI opened pGAPZA (Invitrogen). The resulting vector was called pGAPZAglsII and carries the S. cerevisiae glucosidase II alpha subunit under the transcriptional control of the Pichia GAP promoter. pGAPZAglsII was cut with NsiI,T4/PinAI to isolate a fragment containing the GAP promoter and glsII ORF. The obtained fragment, was ligated into a Sall/PinAI opened pBLADE 1X plasmid creating vector pGAPADE1glsII. Vector pBLADE 1X was a kind gift from Dr Benjamin Glick (Department of Molecular Genetics and Cell Biology, University of Chicago, USA) (Sears et al., 1998) pCR2.1-TOPO3ROT2 was cut with Sall EcoRI and treated with 1µl T4 (Boehringer Mnheim) with 1µl dXTP (10mM) and 1µl of appropriate buffer for 1hr at 37°C. The resulting fragment plasmid was cloned into a T4 treated SalI cut pGAPADE1glsII to yield pGAPADE1glsII3'binv.
A 1222 bp SphI SmaBI URA3 gene fragment of *S. cerevisiae* was cloned into SphI Eco RV opened pGAPADE1glsl3'binv to give pKOROT2.
pGAPADE1glsl3'binv was used as template to introduce the *T. reesei* mutation in the *S. cerevisiae* glucoamylase II gene. The mutagenesis was carried out using

5'GTAGGATCTCCTGCAAAAGCCG3' as mutation sense primer and

5'GACAATACATTGAGGAAAGATCCG3' as mutation antisense primer. The reaction mixture consisted of 80 μl H2O, 10 μl buffer with (NH4)2SO4 – MgCl2, 6 μl MgCl2, 2μl dXTP (10mM), 1 μl mutation sense primer (100 pmol/μl), 1 μl mutation antisense primer (100 pmol/μl), 0.5 μl template DNA and 0.5 μl Taq DNA polymerase. The reaction conditions used were 95°C for 2 min, 94°C for 1 min, 54°C for 1 min, 72°C for 1 min, 24 cycles (from step 2), 72°C for 10 min, cool down till 4°C.

The mutant fragment was reintroduced in pGAPADE1glsl3'binv as a BamHI XcmI fragment and the resulting plasmid was called pGAPADE1GLSIImut3'.

The T4 polymerase treated *EcoO109I* fragment, which contains a LEU2 ORF, of the plasmid YipUTYL was cloned into a T4 treated DraIII/Xhol cut pXY132 to yield pXY132LEU.

The vector pXY132 was purchased from Ingenius (R&D Systems Europe, Abingdon, UK). The vector YipUTYL was taken from the LMB plasmid collection (LMBP 3871).
pGAPADE1GLSIImut3' was cut with *EcoR*I and treated with T4 polymerase, and the GLSII mutant containing fragment was cloned into a *Sma*I treated *Sma*I opened pXY132LEUsite. The resulting plasmid was called pXY132LEUglsl3mut3'.

**N-glycan analysis**

Transformants were grown for 6 days at 30°C, in 100 ml shake flasks containing 50 ml minimal medium with glucose, lactose or cellulose as single carbon source (composition per liter: 20 g dextrose monohydrate or lactose or Solca Flocc cellulose, 5 g (NH4)2SO4, 15 g KH2PO4, 0.3 g CaCl2, 0.3 g MgSO4 and mineral components). N-glycans of the total pool of secreted proteins were prepared according to Papac et al. (1998) from 1 ml of growth medium. The final glycan pellet was resuspended into 5 μl of bidest H2O. 1 μl of this glycan preparation was used for oligosaccharide analysis by DSA-FACE, as described recently (Callewaert, et al., 2001).

Mild acid hydrolysis of the N-glycans was performed on 1 μl of the prepared N-glycan mixture by incubation with 9 μl 10 mM HCl at 100 °C during 30 min. Before DSA-FACE analysis, the sample was dried and the pellet resuspended into 1 μl bidest H2O. *In vitro* α-1,2-mannosidase and β-N-Acetylglucosaminidase digestions were done overnight at 37°C on 1 μl of the prepared N-glycan mixture in 20 mM NaOAc pH 5.0. As enzyme source, in house produced *Trichoderma reesei* α-1,2-mannosidase (Maras et al., 2000) and Jack Bean derived hexosaminidase (Glyko) were used. Before DSA-FACE analysis, the sample was dried and the pellet resuspended into 1 μl bidest H2O.
Analysis of secreted protein
Using shake flask cultures:

*Trichoderma reesei* Rut-C30 WT and transformant g14, expressing a full-size copy of the
1 Trichoderma reesei* glucosidase II alpha subunit, were grown for 6 days at 30°C, in 100 ml
2 shake flasks containing 50 ml of minimal medium with glucose as single carbon source
3 (composition per litter: 20 g dextrose monohydrate, 5 g (NH₄)₂SO₄, 15 g KH₂PO₄, 0.3 g CaCl₂,
4 0.3 g MgSO₄ and mineral components). After growth, the mycelium was separated from
5 the medium and dried overnight at 80°C. Total extracellular protein of a fraction of the growth
6 medium was TCA precipitated. The volume for the different samples taken for the precipitation
7 of the total protein, was normalized against the dry weight of the mycelium. The precipitated
8 proteins were resuspended in loading buffer and analysed by SDS-PAGE. Gels were stained
9 using coomassie brilliant blue (Sigma).

10 Using steady-state growth conditions:

*Trichoderma reesei* strains QM9414, Rut-C30 and its glucosidase II alpha subunit transformant
11 Rut-C30-g31 were grown in steady-state/chemostat conditions. Briefly, the strains were grown
12 at 28°C with a dilution rate of 0.05 h⁻¹. The culture medium consists of 8 g/l lactose, 3.75 g/l
13 KH₂PO₄, 5.7 g/l (NH₄)₂SO₄, 0.17 g/l CaCl₂·2H₂O, 0.375 g/l MgSO₄·7H₂O and 1 ml/l of a trace
14 element solution consisting of 3.7 g/l CoCl₂, 5 g/l FeSO₄·7H₂O, 1.4 g/l ZnSO₄·7H₂O and 1.6 g/l
15 MnSO₄·7H₂O. The pH was kept constant at 5.5: adjustments were done automatically with 0.1
16 N KOH. Foaming was controlled by a mixture of polypropylene glycols. Samples of the
17 chemostat culture were taken at regular time-intervals. Total cellulase activity was measured
18 with para-Nitrophenyl-β-D-lactopyranoside as a substrate and compared to a standard curve of
19 *Trichoderma reesei* cellulases (Sigma). 1 unit releases 1 µmol op-para-Nitrophenol per hour at
20 37°C. Total protein concentration was measured using the Bradford assay, with *Trichoderma
21 reesei* cellulases from Sigma as standard protein.

Analysis of the transformants by lectin screening
Transformants were grown for 6 days at 30°C, in 100 ml shake flasks containing 50 ml minimal
24 medium with glucose as single carbon source. 1 ml of growth medium was used to precipitate
25 the secreted proteins with trichloroacetic acid. Proteins were separated by SDS-PAGE and
26 blotted onto nitrocellulose membranes, using standard techniques (Sambrook et al., 1989).
27 The nitrocellulose membrane was blocked with TNT-buffer (50 mM Tris.HCl pH 7.5; 150 mM
28 NaCl; 0.1% Tween-20) for 1 hour and washed briefly in lectin buffer (50 mM Tris.HCl pH 7.5;
29 150 mM NaCl; 0.05% Tween-20; 1 mM MgCl₂; 1 mM CaCl₂; 1 mM MnCl₂). Afterwards, the
membrane was incubated for 2 hours with a biotinylated Griffonia simplicifolia II lectin, which is specific for terminal GlcNAc (EY laboratories, Inc.). The lectin was diluted in lectin buffer according to the specifications of the provider. The membrane was washed twice (15 minutes in lectin buffer) and incubated for 1 hour in lectin buffer with streptavidin conjugated to peroxidase (Roche). After two wash steps (15 minutes in lectin buffer), the peroxidase was detected using the Renaissance® chemiluminescence kit (NEN™ Life Science). Luminescent signals were captured either using the Lumi-imager™ F1 apparatus from Boehringer Mannheim or on an X-ray film.

IFNβ western blots

Western blots were carried out as described by Redlich and Grossberg (1989) and Grossberg et al., 1986.

IFNβ secretion was tested on a 15% polyacrylamide gel. The primary antibody as an anti-human IFNβ monoclonal antibody (Chemicon International, Temecula, CA, USA). The secondary antibody was a goat anti-mouse HRP conjugated monoclonal anti-IgG1 antibody (Apovia). Visualization was carried out using a Western Lightning Chemiluminescence Reagent Plus kit (Perkin Elmer Life Sciences, Boston, MA, USA).

Bio-informatics

Conversion of nucleotide sequences into amino acid sequences was done using the Translate Tool at http://us.expasy.org/tools/translate. Homology searches were done using the BLAST algorithm at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi. Dual and multiple alignments were performed using the Clustal W algorithm (Thompson et al., 1994) at http://www.ebi.ac.uk/clustalw, resp. the Align program (GENESTREAM network server IGH, Montpellier FRANCE) at http://www2.igh.cnrs.gov/align-quest.cgi (Pearson et al., 1997). General features of the protein (MW, pI, Amino acid composition,...) were assessed using the ProtParam Tool at http://us.expasy.org/tools/protparam.html. The presence of a putative signal sequence was predicted using Signal P (version 1.1) at http://www.cbs.dtu.dk/services/SignalP. Prediction of the presence of transmembrane helices was done using the TMHMM (version 2.0) program at http://www.cbs.dtu.dk/services/TMHMM-2.0 or the HMMTOP (version 2.0) program (by G.E.Tusnady) at http://www.enzim.hu/hmmtop. All above-mentioned tools are either local or accessible via a link on the ExPaSy (Expert Protein Analysis System) proteomics server from the Swiss Institute of Bioinformatics (SIB) (Appel et al., 1994).
**Example 1: Cloning of the Trichoderma reesei glucoamylase II alpha subunit gene.**

**cDNA cloning of the glucoamylase II alpha subunit**

Using the ClustalW algorithm website, an alignment was made between the amino acid sequences of the S. cerevisiae glucoamylase II and the several known mammalian glucoamylase II alpha subunits. Based on several homologous regions, three degenerate primers were designed to screen a cDNA library of the Trichoderma reesei Rut-C30 strain (VTT Biotechnology). Amplification using sense primer 1, antisense primer 3 and the cDNA library as template DNA, resulted into a fragment of the expected size of 1170 bp (figure 3a). Nested PCR amplification including antisense primer 2, resulted into an extra DNA fragment with an expected length of about 970 bp (figure 3b). Both fragments were cloned in the TOPO-TA vector pCR2.1-TOPO (Invitrogen) for sequence analysis. By homology search, the obtained nucleotide sequences proved to be glucoamylase II specific.

Based on this knowledge, cloning of the glucoamylase II alpha subunit cDNA was started from the Rut-C30 cDNA library, using the technique of “cDNA cloning by PCR screening” (Takuma and Lodish, 1994). The PCR analysis was performed using sense primer 1 and antisense primer 3. Each PCR round (three in total) indicated that several wells within the microtiter plate contained at least one glucoamylase II specific clone (figure 4). In the final PCR round each well contained a cell suspension of about 50 different cDNA clones. Two of these wells proved to be positive during the PCR screening. A dilution of the cell suspension of one of those wells was plated on solid Luria Bertani medium. About 200 colonies were streaked on filters for colony hybridization. Using a 32P labeled probe, we identified 7 positive clones (figure 5). DNA of the 7 clones was prepared and analyzed through a XhoI/EcoRI digestion. Fragments of about 1700 bp, 600 bp and 200 bp were obtained and proved to be glucoamylase II specific either by southern hybridization (figure 6) or by sequence analysis after cloning into pUC19 or pBluescript II KS +/- . The completely cloned cDNA fragment consisted of 2290 bp. Homology analysis indicated that a substantial part of the 5' end of the ORF was missing.

**Cloning of the 5' coding sequence of the glucoamylase II alpha subunit**

Both an inverse PCR strategy and a 5' RACE strategy were initiated to clone the missing part. Through inverse PCR on Rut-C30 genomic DNA, a 1700 bp fragment was obtained (figure 7a) and cloned into the pCR2.1-TOPO vector. Partial sequence analysis indicated the presence of two fragments showing homology to the 5' part of the ORF of other known glucoamylase II genes. The two glucoamylase II homologous regions were separated from each other by a 60 bp sequence, containing features that are specific for intron sequences in filamentous fungi. The existence of an intron at the 5' end of the Trichoderma glucoamylase II sequence was further confirmed by a 5' RACE strategy. Using the “First Choice™ RLM-RACE kit (Ambion) we
obtained an 1138 bp fragment (figure 7b) missing the 60 bp intron sequence, but otherwise showing a 100% homology to the already cloned iPCR fragment (figure 7c).

Intron analysis

To evaluate the intron-exon composition of the Trichoderma glucoamylase II alpha subunit gene, a PCR was performed on genomic Rut-C30 DNA using 5' and 3' gene-specific primers. Amplification resulted in a fragment of about 3000 bp, which is close to the length of the coding cDNA. This indicated that only a few, rather small introns could be present. Alignment of the PCR fragment with the cloned cDNA showed that the 60 bp intron at the 5' terminus was the only intron present within the glucoamylase II alpha subunit gene. The small size of this intron is consistent with sizes of most other characterized introns in filamentous fungi (May et al., 1987; Martinez-Blanco et al., 1993; Takaya et al., 1994). The intron follows the GT/AG rule for the 5' and 3' splice site (Mount, 1982). 13 nucleotides upstream of the 3' splice site, the intron contains a lariat sequence of the consensus CTRAC (with R = purine), which is characteristic for other fungal introns (Hiraoka et al., 1984; Orbach et al., 1988; May et al., 1987).

Frame-shift analysis

The DNA sequencing data of the 5' RACE fragment and the cloned cDNA sequence were put together, resulting in a 3621 bp fragment. Translation and BLAST analysis indicated the presence of an ORF showing homology to known glucoamylase II alpha subunits. The glucoamylase II ORF encodes a polypeptide of 807 amino acids. Contrary to the first 695 amino acids, the C-terminal 152 amino acids do not show any considerable sequence homology to other known glucoamylase II alpha subunits. On top of that, the Trichoderma glucoamylase II polypeptide sequence is significantly smaller compared to the yeast or mammalian homologue. This indicated the presence of a frame-shift within the cloned cDNA, resulting into a premature abrogation of translation. Indeed, computer analysis of the 3' 1500 bp of the 3621 bp fragment showed the presence of an out-of-frame sequence of 927 bp encoding a polypeptide of 309 amino acids, which shows high homology to the C-terminus of known glucoamylase II alpha subunits.

Using two glucoamylase II internal primers ROT2TR3_S and ROT2TR0_AS, a fragment of about 320 bp was amplified from the genomic DNA of Trichoderma reeseri Rut-C30 and QM9414. Based on the BLAST homology searches, the annealing sites of the two primers were chosen so that the amplified fragment should contain the site of the frame-shift. Sequence alignment of the two PCR fragments clearly indicates the presence of a frame-shift in the Rut-C30 genome, which was not found within the QM9414 genome: at position 1965 of the glucoamylase II alpha subunit ORF a 'T' is missing. As such, a premature stop codon, 459 nucleotides 3' of the
position of the frame-shift, results in a truncated protein with 153 C-terminal amino acids that are not specific to the *Trichoderma* glucosidase II alpha subunit. This mutation within the Rut-C30 genome could very well explain the difference in glycosylation pattern when comparing the Rut-C30 strain with other *Trichoderma reesei* strains.

5

**General features of the cloned gene**

The knowledge of the nature of the mutation within the glucosidase II alpha subunit gene enabled us to put together some general data on the *Trichoderma* glucosidase II. A full-size non-mutant ORF of 2892 bp encodes a polypeptide of 964 amino acids, which is about the expected length based on data from other known glucosidase II alpha subunits. The protein has a calculated molecular weight of 109,858 Dalton and a theoretical pI of 5.6. Analysis using Signal P (version 1.1) indicated the presence of a putative eukaryotic signal sequence of 30 amino acids. A signal cleavage site was predicted after Leu20Ala30. Analysis of the mature polypeptide sequence with the TMHMM (version 2.0) and the HMMTOP (version 2.0) program did not reveal the presence of transmembrane helices. The polypeptide sequence also seems to lack any known ER-retention signal such as an HDEL tag. These data are in agreement with the general model for the glucosidase II protein: the alpha subunit is the catalytic partner of a heterodimer that is retained within the ER by interacting with the beta subunit, which carries an ER retention signal. The *Trichoderma reesei* glucosidase II alpha subunit has the highest sequence homology (64.2 % identity) to the *Neurospora crassa* counterpart, while the sequence identity with the *Saccharomyces cerevisiae* homologue is only 37.9 %. On the other hand, sequence identity with *Schizosaccharomyces pombe* and higher eukaryotic organisms like pig, human and *Arabidopsis thaliana* is resp. 43.1, 40.9, 40.4 and 40.1 %.

25

**Example 2: Expression of a fully active *Trichoderma* glucosidase II alpha subunit in the Rut-C30 strain:**

**Construction of a *Trichoderma* glucosidase II alpha subunit expression plasmid and transformation to Rut-C30**

A *Trichoderma reesei* expression vector encoding a functional variant of the Rut-C30 glucosidase II alpha subunit was prepared. In a first step, the frame-shift within the cloned cDNA fragment was repaired. In a next step, the 5' RACE fragment and the repaired cDNA were ligated to one another to obtain a full length ORF, encoding a full size and functional alpha subunit. In a last step, the ORF was placed under the transcriptional control of the constitutive gpdA promoter and the TrpC terminator resulting in the vector pFGPDglsITreesel. Using a second strategy, a similar vector was created in which the 10 C-terminal codons of the
ORF were replaced by the coding sequence of the Myc-tag, resulting into vector pFGPDgfslITreseiiMyc.

Both plasmids were transformed to *Trichoderma reesei* Rut-C30 as described by Penttilä et al. (1987) using pAN7.1 as selection plasmid (Punt et al., 1987). Transformants were selected on their resistance to hygromycin. After two rounds of clone purification, single clones were obtained.

**Analysis of the transformants**

The initial analysis of the transformants was based on the functionality of the expressed glucosidase II alpha subunit. For that, transformants were initially grown for 6 days in 50 ml glucose minimal medium, after which the N-glycan profile of the pool of total secreted protein was analyzed. N-glycans were prepared from 0.25 to 1 ml of growth medium as described by Papac and coworkers (1998) and analyzed by DSA-FACE (Callaert et al., 2001a). The N-glycan profiles of the QM9414 strain, which does not carry monoglycosylated N-glycans (Garcia et al., 2001), and the RutC30 WT strain were analyzed in the same way and compared with that of the selected transformants.

Based on the published structural data of the most predominant oligosaccharides synthesized on secreted collobiohydrolase I (De Bruyn et al., 1997, Maras et al., 2000), the profile of the RutC30 strain appeared relatively easy to interpret. An *in vitro* α-1,2-mannosidase digestion was used to characterize the peaks representing monoglycosylated high-mannose glycans. Since the α-1,3-linked glucose blocks the hydrolysis of the two underlying α-1,2-linked mannose residues, a maximum of two mannoses can be released from the glycan substrate, resulting in GlcManα1,2ManNAc2. Mild acid hydrolysis, which hydrolyses phosphodiester linkages, was used to characterize the peaks representing phosphorylated high-mannose carbohydrates. Release of the phosphate-coupled mannose results in a phosphomonoester glycan, which carries an extra negative charge and as such has a higher electrophoretic mobility. Peaks representing these glycans are shifted to the left side of the DSA-FACE profile. As such, using a combination of an α-1,2-mannosidase digestion and a mild acid hydrolysis, the most predominant peaks within the DSA-FACE glycan pattern of the RutC30 strain could be assigned to GlcManα1,2ManNAc2 and their charged counterparts ManPGlcManα1,2GlcNAc2 (figure 8).

A similar analysis was performed on the glycan pattern of QM9414. Initially, the QM9414 DSA-FACE profile looks far more complex. However, comparison with the standard profile of RNase B indicates that a significant fraction of the glycan pool consists of Manα1,2GlcNAc2. This was confirmed via an *in vitro* digestion with α-1,2-mannosidase. Moreover, mild acid hydrolysis of the carbohydrates indicates that most of the peaks at the left hand side of the Manα1,2GlcNAc2 signal represent glycans containing one or more phosphodiester linkages. As such, the
QM9414 glycan peaks could be assigned to neutral and phosphorylated high-mannose N-glycans. The distribution of the phosphorylated N-glycans is not severely changed after α-1,2-mannosidase digestion, since the phosphodiester linkages form a steric hindrance for the enzyme or block the access to underlying α-1,2-linked mannose residues (figure 8).

In a next step, the N-glycan profile of several hygromycin resistant transformants was analyzed. Only one of the analyzed transformants (g14) shows a clear difference in its N-glycan pattern, compared to the RutC30 WT strain. The g14 glycan pool looks more heterogeneous and closer examination indicates that it consists of a combination of the RutC30 and the QM9414 carbohydrate profile (figure 8). Especially at the left hand side of the Man₄GlcNAc₂ peak, a lot of new peaks emerge representing fast migrating oligosaccharides.

Since most of them are susceptible to mild acid hydrolysis, we believe that they represent a structural diversity of phosphorylated high-mannose glycans, analogous to the situation in QM9414. In combination with these charged high-mannose N-glycans, some peaks representing neutral unglycosylated carbohydrates also emerge in the DSA-FACE profile. The presence of these structures was further confirmed by performing an in vitro α-1,2-mannosidase digestion. Comparison of the g14 with the RutC30 profile however, clearly indicates that still a significant amount of monoglycosylated glycans (neutral and charged GlcMan₃α(GlcNAc)₂) is synthesized on the proteins of the transformed strain. Presumably, the amount of full-size glucosidase II is not sufficient to hydrolyze all α-1,3-linked glucose residues.

A number of hygromycin resistant transformants, including g14, and the WT RutC30 strain were analyzed on the genomic level. To clearly discriminate between endogenous mutant alpha subunit locus and the repaired cDNA from the integrated expression vector, genomic DNA was digested with KpnI/Nhel (Nhel cuts within the gpdA promoter of the expression vector) and analysed via Southern blot using a glucosidase II specific probe. Using this strategy, two bands of approximately 5000 and 3400 bp were visualized for the g14 transformant, the latter one resulting from a random integration of the alpha subunit expression cassette into the Trichoderma reesei genome. For all other transformants showing no change in their N-glycan profile, only the fragment of about 5000 bp was identified. This is identical to the band obtained for the untransformed RutC30 strain and as such can only result from a hybridisation event to the endogenous locus encoding the truncated Glc.

Effect of the overexpression of the glucosidase II alpha subunit on the secretion capacity of the transformants

The Trichoderma reesei Rut-C30 strain, which is a hypersecretor of cellulases, synthesizes unusual N-glycan structures on its secreted proteins. Thorough analysis indicated that most of the oligosaccharides carries α-1,3-glucose residues (Maras et al. 1997). These capping structures, which prevent further trimming of the glycans by α-1,2-mannosidase, are probably
the result of an inefficient glucosidase II activity. Several events may cause this phenomenon: (1) the glucosidase II simply cannot cope the rich load of protein passing through the secretion pathway during cellulase-inducing conditions; (2) during the consecutive rounds of mutagenesis leading to this hypersecretor strain, one or more mutations have occurred within the glucosidase II gene or its transcriptional elements.

Surprisingly, we found that restoration of the glucosidase II activity affects the secretion capacity that is similar to that of the wild type strain. Transformant g14, where both glucosidase II forms are expressed, shows a secretion capacity that is significantly lower than that of the hypersecreting strain Rul-C30, clearly indicating that the glucosidase II plays an important role in the level of secretion.

Example 3: Expression of α-1,2-mannosidase in Trichoderma

In order to localize most of the recombinant α-1,2-mannosidase to the ER compartment, where it can act on the substrate Man₆GlcNAc₂ to deliver a Man₅GlcNAc₂ structure, an HDEL-tag was added at the C-terminal end of the protein. By doing so the recombinant protein is recycled in a COP1 dependent manner from the Golgi apparatus to the ER due to binding to the HDEL-receptor.

An expression cassette containing the constitutive gapA promoter, the prepro-signal sequence of the yeast α-mating factor for directing the protein into the secretory pathway, the HDEL-tagged α-1,2-mannosidase ORF and the trpC terminator was constructed. The construct was transformed using AmdS (acetamidase) as a selection marker. Transformants were selected for their ability to grow on minimal medium with acetamide as a sole nitrogen source. The transformants were submitted to several selection rounds in order to get single or "pure" clones.

To assess the functionality of the ER-localized α-1,2-mannosidase, the N-glycan profile of the total pool of secreted protein was analyzed. For this, transformants were grown in glucose containing minimal medium as described in materials and methods. N-glycans were released from 1 ml of growth medium through the method described by Papac et al., 1998. The results from the DSA-FACE analysis are depicted in figure 12. In total, 16 transformants were analyzed by DSA-FACE, of which 4 had the expected Man₅GlcNAc₂ glycan pattern. Evidence for the presence of the mannosidase expression plasmid in the genome of the transformants was obtained by Southern blotting/PCR analysis. Only a very small amount of the total glycan pool consists of Man₆GlcNAc₂, some of them carrying mannosephosphate. An additional in vitro digestion with purified recombinant α-1,2-mannosidase almost completely converts the remnant neutral high-mannose N-glycans to Man₅GlcNAc₂.

When grown in glucose minimal medium, the total amount of secreted cellulases is rather low. To evaluate the trimming capacity of the ER-localized α-1,2-mannosidase during cellulase
inducing conditions, one of the positive transformants (transformant F4) was grown in minimal medium with 2% lactose, 5% lactose or SolcsFlc cellulose as C-source instead of 2% glucose. N-glycan analysis was as described in materials and methods. When comparing the N-glycan profiles from the different growth conditions, we found almost no difference. This suggests that the recombinant α-1,2-mannosidase is expressed in sufficient amounts to deal with a large flow of protein within the secretory pathway. The results clearly indicate that the ER localization of a functional α-1,2-mannosidase enables the fungus to convert most of the ER high mannose structure Man_{α6}GlcNAc_{2} to Man_{α3}GlcNAc_{2}. By doing so, phosphomannosylation of the N-glycans is almost completely abolished. It seems that in the untransformed strain, the phosphomannosyltransferase and the α-1,2-mannosidase compete for the same high-mannose oligosaccharides. The obtained Man_{α3}GlcNAc_{2} structure is no substrate for the phosphomannosyltransferase, which is in accordance with data published for the S. cerevisiae Mnn6p transferase protein (Wang et al, 1997). This also suggests that the Trichoderma phosphomannosyltransferase activity is located somewhat further in the secretion pathway (medial to trans Golgi).

In conclusion, the obtained results indicate that by using this strategy, we can convert the fungal-type N-glycosylation pattern of Trichoderma reesii to almost exclusively Man_{α3}GlcNAc_{2}. Since this is the substrate for the GlcNAc transferase I, the key enzyme in the synthesis of complex N-glycans, new possibilities in creating a Trichoderma strain with a more human-like glycan profile can be explored.

**Example 4: expression of GlcNAc-transferase in Trichoderma**

For GlcNAc-transferase I to be localized to the Golgi compartment, where it can act on the Man_{α3}GlcNAc_{2} structure, two chimeras were created in the past between the C-terminal part of GlcNAc-transferase I and the N-terminal part of yeast Kre2, a Golgi localized mannosyltransferase (Lussier et al., 1995). The fusion positions were based on the fact that both proteins contain a putative coiled coil that might be important for localization and oligomerisation of the protein. Indeed, when the respective amino acid sequences were analyzed by the paircoil program (Berger et al., 1995), a coiled coil was predicted from amino acid 49 to 81 in GlcNAc-transferase I with a probability of 0.36 and from amino acid 54 to 99 in Kre2 with a probability of maximum 0.69 (see also figure 14). In addition, when analyzing the GlcNAc transferase I of other organisms, the probability of the presence of a coiled coil structure was even higher. Based on results obtained in Aspergillus niger, plasmid pFGPDKre2GmTl encoding a chimer of the first 100 amino acids of Kre2 and the C-terminal part of GlcNAc-transferase I starting from amino acid 103 (as such having the coiled coil of Kre2), was preferred for the expression of recombinant human GlcNAc transferase I in Trichoderma reesii.
The construct was co-transformed to the α-1,2-mannosidase expressing transformant F4 of strain QM9414. The *Streptosporaleichus hindustanus* phleomycin-binding protein expression cassette was used as selection marker. Transformants were selected based on their capacity to grow on minimal medium containing zeocin. The transformants were submitted to several selection rounds in order to get single or “pure” clones.

To identify functional GlcNAc-transferase I transformants a first screening round was performed using the *Griffonia simplicifolia* II lectin, which is specific for terminal GlcNAc residues. Several transformants were grown for 6 days on glucose minimal medium. Transformants that score positive during the lectin screening were further analyzed by DSA-FACE. N-glycans were released from 1 ml of growth medium through the method described by Papac et al., 1998. Changes in the glycosylation profile that could indicate the functional expression of GlcNAc transferase I were investigated by *in vitro* digestion of with Jack Bean hexosaminidase (Glyko): strains of which the pattern returned to the Man₆GlcNAc₂ profile after the *in vitro* digestion, proof to be the desired glycosylation transformants.

Example 5: Effect of the overexpression of the glucosidase II alpha subunit on the secretion capacity of the transformants

The *Trichoderma reesei* RutC30 strain contains a frame-shift mutation in the glucosidase II alpha subunit gene, resulting in the production of a partially defective gene product. To restore the normal ER-processing of protein-linked N-glycans, this strain was transformed with the expression plasmid pFGPDPgsIIrTeeseI (Figure 2), containing the full-size *Trichoderma* glucosidase II alpha subunit (GIIα) gene under the transcriptional control of the constitutive gpdA promoter. Transformation was done according to Penttilä and coworkers (1987). Vector pAN7.1 (Punt et al., 1987) was co-transformed to enable selection of the transformants on hygromycin containing medium.

Several hygromycin-resistant clones were analysed by DSA-FACE. As described in example 2, only one transformant (designated as g14) showed a severe difference in its N-glycan profile compared to the RutC30 untransformed strain. Southern analysis indicated that only g14 had randomly integrated the GIIα expression plasmid into its genome (figure 9).

Apart from the phenotype on the N-glycan level, also the secretion capacity of the g14 transformant seems to be affected. To analyse the effect on the production of extracellular proteins, several strains were grown on 50 ml minimal medium in 100 ml shake flasks. Incubations were performed for 6 to 7 days, at 30°C and 150 rpm (rotations per minute). The minimal medium consists per liter of 20 g glucose, 5 g (NH₄)₂SO₄, 15 g KH₂PO₄, 0.3 g CaCl₂, 0.3 g MgSO₄ and mineral components. Since all analyzed clones are derived from the RutC30 strain, their cellulose expression is not subject to carbon catabolite repression due to the absence of a functional CRE1 (Ilimen, et al., 1996). Hence, a sufficient amount of extracellular
hydrolysis is synthesized to perform an SDS-PAGE analysis. The proteins are precipitated from the growth medium using TCA (trichloro-acetic acid), resuspended in 2x Laemmli loading buffer and analysed via gel electrophoresis (Figure 15). Interpretation of the observed SDS-PAGE profile indicates that the secretion capacity of the g14 transformant is reduced compared to the RutC30 WT and untransformed strains. The exercise was repeated several times to check the reproducibility of the obtained data. After growth on glucose minimal medium for 6 to 7 days, the level of the g14 secretion seems to be lower than that of the RutC30 strain.

Example 8: construction of a glucosidase II knock out in Saccharomyces cerevisiae
The strategy to construct the ROT2 knock out is summarized in Figure 16. pKOROT2 is a vector comprising an integration cassette consisting of a S. cerevisiae URA3 expression cassette inserted about 60 bp of the 5’ end of the ROT2 ORF at one side and the 3’ end untranslated region of this ORF at the other side. The plasmid pKOROT2 was digested with Xhol to release the integration cassette. Transformation of S. cerevisiae YA-72 with this cassette and selection on a URA’ medium results in the selection of mutants in which the yeast glucosidase II gene has been replaced by the URA3 expression cassette. Transformants were tested using an upward primer in the URA3 gene and a downward primer in the 3’ untranslated region. 3 out of 19 analyzed clones were showing the right insert. The positive clones were tested on their sugar profile using DSA-FACE analysis, and compared with a negative clone, with the parental strain and with the rot2 knock out mutant Y13369 and its parental strain BY4742 (Figure 17). From the sugar profiles, it can indeed be concluded that the glucosidase II genes was inactivated in the transformants. Figure 16 indicates how the URA3 gene in the knock out further can be exchanged against a mutant glucosidase II gene, carrying the mutation that is found in the T. reesei Rut C30 glucosidase II.

Example 7: S. cerevisiae strains with a mutant glucosidase II show increased secretion
S. cerevisiae Y13369, as well as the parental strain BY4742 were transformed with the episomal plasmid pSCGALMFI:N3B, carrying the human IFNβ gene preceded by the S. cerevisiae mating factor and under control of the GAL1 promoter. Transformants were selected on URA’ medium. From both strain, 8 transformants were analyzed by Western Blotting. The yeast strains were precultivated for 48 hours in YPD, and the expression was induced for 48 hours in YPGal. The proteins, secreted in the medium were precipitated with TCA and separated using a 15% SDS-PAGE gel. Blotting was carried out by the semi-dry method, and the results are summarized in Figure 18.
Although the results are not quantified, it is clear that in general the knockout mutants do secrete more IFNβ in the medium than the wild type strain.

To obtain more quantitative data, the experiment was repeated and the secretion was compared with the secretion of an IFNβ producing knock out complemented with a mutant glucosidase. This strain was obtained by transforming the IFNβ knock out strain with pYX132LEUGLSlmut3' and selection of SDC URA LEU medium.

8 individual transformants of each strain (Y13369 transformed with pSCGALMFWFBNB2, BY4742 transformed with pSCGALMFWFBNB2 and Y13369 transformed with both pSCGALMFWFBNB2 and pYX132LEUGLSlmut3') were grown in selective medium (SDC URA or SDC URA LEU') for 18 hours. Then the cells were harvested and washed three times with water. The expression was induced by resuspending the cells in SDGal URA, resp. SDGal URA LEU and cultivating them for another 24 hours. The cells were pelleted and the medium was collected. The supernatant of each of the 8 transformants was pooled. Two samples of the pooled supernatant, one of 0.5 ml and one of 1 ml was TCA precipitated. The-proteins were separated using an SDS-PAGE gel, and blotted as described above. The results are summarized in Figure 19 and Table 1. It is clear from these results that both the mutant glucosidase II and the knock out mutant show an increased secretion compared with the wild type.

Table 1: Quantification of the protein bands of Figure 19, ad determined by Lumi Imager. A: analysis of the 0.5ml sample. B: analysis of the 1ml sample. The values are expressed as relative intensity ratios.

<table>
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References


Claim:

1. The use of a glucosidase II mutation to increase protein secretion.
2. The use according to claim 1, whereby said mutation is an inactivating knock out mutation.
3. The use according to claim 1, whereby said mutation is a point mutation.
4. The use according to claim 1-3 whereby said mutation affects only one of the subunits of the enzyme.
5. The use according to claim 4, whereby said mutation affects subunit alpha.
6. The use according to any of the claims 1-5, whereby said mutation is carried out in a eukaryotic cell.
7. The use according to claim 6, whereby said eukaryotic cell is a fungal cell.
8. The use according to claim 7, whereby said fungal cell is a yeast cell.
9. The use according to claim 6, whereby said yeast cell is a Saccharomyces sp.
10. The use according to claim 6, whereby said fungal cell is a filamentous fungus.
11. The use according to claim 10, whereby said filamentous fungus is selected from the group genera consisting of the genera Aspergillus, Fusarium, Geotrichum, Monascus, Monilia, Mucor, Penicillium, Rhizopus, Trichoderma and Ustilago.
12. The use according to claim 10, whereby said filamentous fungus is a Trichoderma sp.
13. The use of a filamentous fungus comprising a recombinant defective glucosidase II as a host for protein secretion.
14. The use according to claim 13 whereby said filamentous fungus is selected from the group genera consisting of the genera Aspergillus, Fusarium, Geotrichum, Monascus, Monilia, Mucor, Penicillium, Rhizopus, Trichoderma and Ustilago.
15. The use according to claim 12 whereby said filamentous fungus is a Trichoderma sp.
16. The use of a yeast comprising a defective glucosidase II as host for protein secretion.
17. The use according to claim 16 whereby said defective glucosidase II is a recombinant glucosidase II.
18. The use according to claim 16 or 17 whereby said yeast is selected from the group consisting of Kluyveromyces sp., Pichia sp., Hansenula sp. or Schizosaccharomyces pombe.
19. The use according to claim 16 or 17 whereby said yeast is a Saccharomyces sp.
20. The use according to any of the claims 13 to 19 whereby said glucosidase is defective in subunit alpha.
21. A method to increase protein secretion of a eukaryotic cell, comprising mutagenesis of glucosidase II.
Fig. 1:

```
BamHI

Genome Unknown piece

Nco I

Primer 1 Primer 2

Known piece of glucosidase II

BamHI
```
Fig. 2C:

Mutagenesis insertion of Xmal/Smal site

EcoRI/SalI

HindIII/NcoI-S1

HindIII/SacII-T4

SmaI/SnaBI

NcoI-S1/Bsp120I-Klenow

Mrd

4/20
Fig. 3:

A

1164 bp

B

-1164 bp
-969 bp
Fig. 7:

In reverse PCR: A
5' RACE: ATGAGG

In 5' RACE: ATGAGG

In reverse PCR: B
5' RACE: ATTTCC

In 5' RACE: ATTTCC

In reverse PCR: C
5' RACE: CAGGCG

In 5' RACE: CAGGCG

In reverse PCR: D
5' RACE: ACCCTG

In 5' RACE: ACCCTG
Fig. 8:

**Glucose units**

![Glucose units diagram](image-url)
Fig. 9:

RutC30

M9  G1  G14  Ref  G20  G21  G31  G46  G2
Fig. 10:

NcoI - Mung Bean nuclease
NotI

BstBI - T4 polymerase
NotI
Fig. 14:

A

B
Fig. 16:

**Wild type**

**Knock-out**

**Mutant 1**

**Mutant 2**
Fig. 18:

1 2 3 4 5 6 7 8 A B C D M E F G H
Fig. 19:

M  C2  C1  mut  KO  WT  M

M  WT  KO  mut  C1  C2  M