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(71) Applicant *(for all designated States except US)*: **CONCORDIA UNIVERSITY** [CA/CA]; 1550 de Maisonneuve Blvd. West, Suite GM 1000, Montreal, Quebec H3G 1M8 (CA).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SILLAOTS, Susan** [CA/CA]; 5401 MacDonald Avenue, Montreal, Quebec H3X 2W6 (CA). **MARTINEZ-PEREZ, Amalia** [CA/CA]; 243 Daker Cr., Greenfield Park, Quebec J4V 2X1 (CA). **TSANG, Adrian** [CA/CA]; 3816 Draper Avenue, Montreal, Quebec H4A 2P1 (CA). **STORMS, Reginald** [CA/CA]; 97 Prince, Beaconsfield, Quebec H9W 3M8 (CA).

(74) Agents: **DUBUC, Jean, H.** et al.; Goudreau Gage Dubuc, The Stock Exchange Tower, Suite 3400, 800 Place Victoria, P.O. Box 242, Montreal, Quebec H4Z 1E9 (CA).

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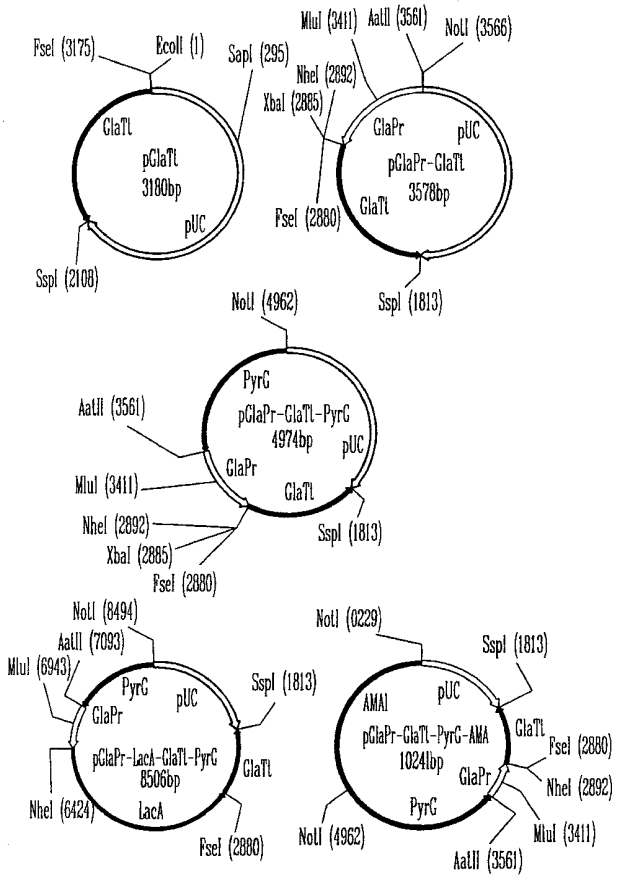

(57) Abstract: The present invention relates to expression modules that can be used to assemble integrating and autonomously replicating vectors for use with filamentous fungi. The vectors constructed with these modules are all based on the backbone of the *E. Coli* plasmid pUC18. They can therefore be used to perform most standard DNA manipulations in the same plasmid that is introduced into the fungus. This system of vectors can be used to study promoter sequences, express heterologous and homologous proteins, and identify vector and host strain mutations that alter protein expression. A subset of these vectors, which include the *Aspergillus niger LacA* coding region as a reporter gene, were used to develop a high-throughput indicator plate-based screen that could identify host and vector mutants that increase the expression of the secreted reporter protein, LacAp. Finally, the vectors and strains developed can be used to develop strains that can express the coding region for essentially any homologous or heterologous protein at high levels.

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TITLE OF THE INVENTION

A MULTIFUNCTIONAL SYSTEM FOR THE EFFICIENT MANIPULATION OF PROTEIN EXPRESSION IN FILAMENTOUS FUNGI AND METHOD USING SAME

FIELD OF THE INVENTION

The present invention relates to filamentous fungi. More particularly, the invention relates to a multifunctional system for the efficient manipulation of protein expression in filamentous fungi and method using same. The invention also relates to plasmid vectors adapted for expression in filamentous fungi. In addition, the invention relates to methods to increase the efficiency and reduce the time required for isolating strains that express proteins of interest at high levels, and to such strains.

BACKGROUND OF THE INVENTION

Relative to the extensive assortment of plasmid vectors available for use in model systems like yeast and *E. coli,* comparably few vectors have been developed for use in filamentous fungi. Most of these have been developed for basic research using the more popular laboratory systems *Neurospora crassa* and *Emericella nidulans*. There are therefore very few plasmid vectors available for studies with fungi like *A. niger* and *A. oryzae*, organisms that have not been exploited extensively for basic research but which are widely used industrially for the production of organic acids and secreted proteins. More specifically, expression vectors for the systematic analysis of gene expression and high-throughput strain improvement screens with industrially important filamentous fungi are not generally available.

Gene expression in eukaryotic cells is often studied by linking a promoter sequence to an easily detectable "reporter" gene. Examples of such "reporter" genes include firefly luciferase, and *E. coli* chloramphenicol acetyltransferase (CAT), ß-galactosidase and ß-glucuronidase. Reporter genes

simplify gene expression studies by "reporting" upstream transcriptional events through the expression of an easily assayable reporter protein. Typically, reporter genes monitor regulatory DNA associated with promoters (cis-acting sequences) and DNA-binding proteins (trans-acting factors). In addition to gene regulation studies, reporter genes are used in a variety of applications including studies of protein-protein interactions (Fields and Song, 1989), recombination events (Herzing et al., 1993), gene targeting (Vile et al., 1993), RNA processing (Huang et al., 1990), signal transduction (Medema et al., 1992 and Sakoda et al., 1992) and transformation efficiency (Takahashi et al 1991, and Hall et al. 1992). To facilitate reporter gene based studies in filamentous fungi, a repertoire of practical reporter vectors and a robust assay would be invaluable.

Filamentous fungi like *A. niger* are often used as expression systems to produce commercially valuable proteins. Because high levels of expression are important it is often necessary to screen for mutant isolates that express desirable proteins more efficiently. Traditionally mutants with improved characteristics have been isolated by screening methods that involve costly and time consuming assays performed on tens of thousands of mutagenized colonies. It would therefore be desirable to develop high-throughput screening methods that increase the efficiency and reduce the time required for isolating strains that express the protein of interest at high levels.

The present description refers to a number of documents, the content of which is herein incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

The present invention concerns a set of expression vectors for studying gene expression in filamentous fungi in general and demonstrates that these vectors can be used to isolate strains that express secreted proteins at the very high levels required for industrial-scale protein production.

More specifically, the invention pertains to the filamentous fungi *A. niger*.

The expression vectors constructed fall into two classes, integrating plasmids and autonomously replicating plasmids. The utility of these vectors for studying gene expression has been dramatically facilitated by including a reporter gene that can be assayed easily. For industrial applications it is often desirable to secrete the protein of interest into the growth medium. To address this we report herein the successful utilization of the ß-galactosidase coding *LacA* gene from *A. niger* as a reporter. The encoded lactase is easily assayed and efficiently secreted, making it ideal for assessing gene expression and protein secretion in *A. niger.* The *LacA* reporter was successfully used in a high-throughput indicator plate-based screen to isolate strains with improved protein expression characteristics. It was also used to engineer derivatives of the *GlaA* promoter which directed dramatically increased levels of expression. By combining our improved strains and vectors we have increased the expression of secreted ß-galactosidase to greater than 1000 times the levels expressed by the parent strain (1.1 grams per liter versus 0.001 grams per liter).

The present invention also relates to an expression vector capable of enabling a systematic analysis of gene expression and/or high-throughput strain improvement screens in a filamentous fungi comprising:

a) a selectable marker for said filamentous fungi; and

b) a promoter operably linked to a nucleic acid sequence encoding a protein of interest or part thereof.

In addition, the present invention relates to a filamentous fungi strain, enabling the expression of a protein of interest to greater than about 100 times, and preferably greater than about 1000 times, as compared to the level thereof in parent strain of the filamentous fungi strain.

In order to provide a clear and consistent understanding of terms used in the present description, a number of definitions are provided hereinbelow.

Nucleotide sequences are presented herein by single strand, in the 5' to 3' direction, from left to right, using the one letter nucleotide symbols as commonly used in the art and in accordance with the recommendations of the IUPAC-IUB Biochemical Nomenclature Commission.

Unless defined otherwise, the scientific and technological terms and nomenclature used herein have the same meaning as commonly understood by a person of ordinary skill to which this invention pertains. Generally, the procedures for cell cultures, infection, molecular biology methods and the like are common methods used in the art. Such standard techniques can be found in reference manuals such as for example Sambrook et al. (1989, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratories) and Ausubel et al. (1994, Current Protocols in Molecular Biology, Wiley, New York).

The present description refers to a number of routinely used recombinant DNA (rDNA) technology terms. Nevertheless, definitions of selected examples of such rDNA terms are provided for clarity and consistency.

As used herein, "nucleic acid molecule", refers to a polymer of nucleotides. Non-limiting examples thereof include DNA (e.g. genomic DNA, cDNA) and RNA molecules (e.g. mRNA). The nucleic acid molecule can be obtained by cloning techniques or synthesized. DNA can be double-stranded or single-stranded (coding strand or non-coding strand [antisense]).

The term "recombinant DNA" as known in the art refers to a DNA molecule resulting from the joining of DNA segments. This is often referred to as genetic engineering.

The term "DNA segment", is used herein, to refer to a DNA molecule comprising a linear stretch or sequence of nucleotides. This sequence when read in accordance with the genetic code, can encode a linear stretch or sequence of amino acids which can be referred to as a polypeptide, protein, protein fragment and the like.

The terminology "amplification pair" refers herein to a pair of oligonucleotides (oligos) of the present invention, which are selected to be used together in amplifying a selected nucleic acid sequence by one of a number of types of amplification processes, preferably a polymerase chain reaction. Other types of amplification processes include ligase chain reaction, strand displacement amplification, or nucleic acid sequence-based amplification, as explained in greater detail below. As commonly known in the art, the oligos are designed to bind to a complementary sequence under selected conditions.

The nucleic acid (e.g. DNA or RNA) for practicing the present invention may be obtained according to well-known methods.

Oligonucleotide probes or primers of the present invention may be of any suitable length, depending on the particular assay format and the particular needs and targeted genomes employed. In general, the oligonucleotide probes or primers are at least 12 nucleotides in length, preferably between 15 and 24 molecules, and they may be adapted to be especially suited to a chosen nucleic acid amplification system. As commonly known in the art, the oligonucleotide probes and primers can be designed by taking into consideration the melting point of hybridization thereof with its targeted sequence (see below and in Sambrook et al., 1989, Molecular Cloning - A Laboratory Manual, 2nd Edition, CSH Laboratories; Ausubel et al., 1989, in Current Protocols in Molecular Biology, John Wiley & Sons Inc., N.Y.).

The term "DNA" molecule or sequence (as well as sometimes the term "oligonucleotide") refers to a molecule comprised of the deoxyribonucleotides adenine (A), guanine (G), thymine (T) and/or cytosine (C), in a double-stranded form, and comprises or includes a "regulatory element" according to the present invention, as the term is defined herein. The term "oligonucleotide" or "DNA" can be found in linear DNA molecules or fragments, viruses, plasmids, vectors, chromosomes or synthetically derived DNA. As used

herein, particular double-stranded DNA sequences may be described according to the normal convention of giving only the sequence in the 5' to 3' direction.

"Nucleic acid hybridization" refers generally to the hybridization of two single-stranded nucleic acid molecules having complementary base sequences, which under appropriate conditions will form a thermodynamically favored double-stranded structure. Examples of hybridization conditions can be found in the two laboratory manuals referred above (Sambrook et al., 1989, *supra* and Ausubel et al., 1989, *supra*) and are commonly known in the art. In the case of a hybridization to a nitrocellulose filter, as for example in the well-known Southern blotting procedure, a nitrocellulose filter can be incubated overnight at 65°C with a labeled probe in a solution containing 50% formamide, high salt (5 x SSC or 5 x SSPE), 5 x Denhardt's solution, 1% SDS, and 100 µg/ml denatured carrier DNA (e.g. salmon sperm DNA). The non-specifically binding probe can then be washed off the filter by several washes in 0.2 x SSC/0.1% SDS at a temperature which is selected in view of the desired stringency: room temperature (low stringency), 42°C (moderate stringency) or 65°C (high stringency). The selected temperature is based on the melting temperature (Tm) of the DNA hybrid. Of course, RNA-DNA hybrids can also be formed and detected. In such cases, the conditions of hybridization and washing can be adapted according to well-known methods by the person of ordinary skill. Stringent conditions will be preferably used (Sambrook et al.,1989, *supra*).

Probes of the invention can be utilized with naturally occurring sugar-phosphate backbones as well as modified backbones including phosphorothioates, dithionates, alkyl phosphonates and α-nucleotides and the like. Modified sugar-phosphate backbones are generally taught by Miller, 1988, Ann. Reports Med. Chem. 23:295 and Moran et al., 1987, Nucleic Acids Res., 14:5019. Probes of the invention can be constructed of either ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), and preferably of DNA.

The types of detection methods in which probes can be used include Southern blots (DNA detection), dot or slot blots (DNA, RNA), and Northern blots (RNA detection). Although less preferred, labeled proteins could also be used to detect a particular nucleic acid sequence to which it binds. Other detection methods include kits containing probes on a dipstick setup and the like.

Although the present invention is not specifically dependent on the use of a label for the detection of a particular nucleic acid sequence, such a label might be beneficial, by increasing the sensitivity of the detection. Furthermore, it enables automation. Probes can be labeled according to numerous well-known methods (Sambrook et al., 1989, supra). Non-limiting examples of labels include ^{3}H, ^{14}C, ^{32}P, and ^{35}S. Non-limiting examples of detectable markers include ligands, fluorophores, chemiluminescent agents, enzymes, and antibodies. Other detectable markers for use with probes, which can enable an increase in sensitivity of the method of the invention, include biotin and radionucleotides. It will become evident to the person of ordinary skill that the choice of a particular label dictates the manner in which it is bound to the probe.

As commonly known, radioactive nucleotides can be incorporated into probes of the invention by several methods. Non-limiting examples thereof include kinasing the 5' ends of the probes using gamma ^{32}P ATP and polynucleotide kinase, using the Klenow fragment of Pol I of *E. coli* in the presence of radioactive dNTP (e.g. uniformly labeled DNA probe using random oligonucleotide primers in low-melt gels), using the SP6/T7 system to transcribe a DNA segment in the presence of one or more radioactive NTP, and the like.

As used herein, "oligonucleotides" or "oligos" define a molecule having two or more nucleotides (ribo or deoxyribonucleotides). The size of the oligo will be dictated by the particular situation and ultimately on the particular use thereof and adapted accordingly by the person of ordinary skill. An

oligonucleotide can be synthesized chemically or derived by cloning according to well-known methods.

As used herein, a "primer" defines an oligonucleotide, which is capable of annealing to a target sequence, thereby creating a double stranded region, which can serve as an initiation point for DNA synthesis under suitable conditions.

Amplification of a selected, or target, nucleic acid sequence may be carried out by a number of suitable methods. See generally Kwoh et al., 1990, Am. Biotechnol. Lab. 8:14-25. Numerous amplification techniques have been described and can be readily adapted to suit particular needs of a person of ordinary skill. Non-limiting examples of amplification techniques include polymerase chain reaction (PCR), ligase chain reaction (LCR), strand displacement amplification (SDA), transcription-based amplification, the Qβ replicase system and NASBA (Kwoh et al., 1989, Proc. Natl. Acad. Sci. USA86, 1173-1177; Lizardi et al., 1988, BioTechnology 6:1197-1202; Malek et al., 1994, Methods Mol. Biol., 28:253-260; and Sambrook et al., 1989, *supra*). Preferably, amplification will be carried out using PCR.

Polymerase chain reaction (PCR) is carried out in accordance with known techniques. See, e.g., U.S. Pat. Nos. 4,683,195; 4,683,202; 4,800,159; and 4,965,188 (the disclosures of all three U.S. Patent are incorporated herein by reference). In general, PCR involves, a treatment of a nucleic acid sample (e.g., in the presence of a heat stable DNA polymerase) under hybridizing conditions, with one oligonucleotide primer for each strand of the specific sequence to be detected. An extension product of each primer, which is synthesized, is complementary to each of the two nucleic acid strands, with the primers sufficiently complementary to each strand of the specific sequence to hybridize therewith. The extension product synthesized from each primer can also serve as a template for further synthesis of extension products using the same primers. Following a sufficient number of rounds of synthesis of extension

products, the sample is analyzed to assess whether the sequence or sequences to be detected are present. Detection of the amplified sequence may be carried out by visualization following EtBr staining of the DNA following gel electrophores, or using a detectable label in accordance with known techniques, and the like. For a review on PCR techniques (see PCR Protocols, A Guide to Methods and Amplifications, Michael et al. Eds, Acad. Press, 1990).

As used herein, the term "gene" is well-known in the art and relates to a nucleic acid sequence defining a single protein or polypeptide. A "structural gene" defines a DNA sequence which is transcribed into RNA and translated into a protein having a specific amino acid sequence thereby giving rise to a specific polypeptide or protein. It will be readily recognized by the person of ordinary skill, that the nucleic acid sequence of the present invention can be incorporated into anyone of numerous established kit formats, which are well-known in the art.

A "heterologous" (e.g. a heterologous gene) region of a DNA molecule is a subsegment of DNA within a larger segment that is not found in association therewith in nature. The term 'heterologous" can be similarly used to define two polypeptidic segments not joined together in nature. Non-limiting examples of heterologous genes include reporter genes such as luciferase, chloramphenicol acetyl transferase, β-galactosidase, and the like which can be juxtaposed or joined to heterologous control regions or to heterologous polypeptides.

The term "vector" is commonly known in the art and defines a plasmid DNA, phage DNA, viral DNA and the like, which can serve as a DNA vehicle into which DNA of the present invention can be cloned. Numerous types of vectors exist and are well-known in the art.

The term "expression" defines the process by which a gene is transcribed into mRNA (transcription), the mRNA is then being translated (translation) into one polypeptide (or protein) or more.

The terminology "expression vector" defines a vector or vehicle as described above but designed to enable the expression of an inserted sequence following transformation into a host. The cloned gene (inserted sequence) is usually placed under the control of control element sequences such as promoter sequences. The placing of a cloned gene under such control sequences is often referred to as being operably linked to control elements or sequences.

Operably linked sequences may also include two segments that are transcribed onto the same RNA transcript. Thus, two sequences, such as a promoter and a "reporter sequence" are operably linked if transcription commencing in the promoter will produce an RNA transcript of the reporter sequence. In order to be "operably linked" it is not necessary that two sequences be immediately adjacent to one another.

Expression control sequences will vary depending on whether the vector is designed to express the operably linked gene in a prokaryotic or eukaryotic host or both (shuttle vectors) and can additionally contain transcriptional elements such as enhancer elements, termination sequences, tissue-specificity elements, and/or translational initiation and termination sites.

Prokaryotic expressions are useful for the preparation of large quantities of the protein encoded by the DNA sequence of interest. This protein can be purified according to standard protocols that take advantage of the intrinsic properties thereof, such as size and charge (e.g. SDS gel electrophoresis, gel filtration, centrifugation, ion exchange chromatography...). In addition, the protein of interest can be purified via affinity chromatography using polyclonal or monoclonal antibodies. The purified protein can be used for therapeutic applications.

The DNA construct can be a vector comprising a promoter that is operably linked to an oligonucleotide sequence of the present invention,

which is in turn, operably linked to a heterologous gene, such as the gene for the luciferase reporter molecule. "Promoter" refers to a DNA regulatory region capable of binding directly or indirectly to RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of the present invention, the promoter is bound at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter will be found a transcription initiation site (conveniently defined by mapping with S1 nuclease), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CCAT" boxes. Prokaryotic promoters contain -10 and -35 consensus sequences, which serve to initiate transcription and the transcript products contain Shine-Dalgarno sequences, which serve as ribosome binding sequences during translation initiation.

As used herein, the designation "functional derivative" denotes, in the context of a functional derivative of a sequence whether a nucleic acid or amino acid sequence, a molecule that retains a biological activity (either function or structural) that is substantially similar to that of the original sequence. This functional derivative or equivalent may be a natural derivative or may be prepared synthetically. Such derivatives include amino acid sequences having substitutions, deletions, or additions of one or more amino acids, provided that the biological activity of the protein is conserved. The same applies to derivatives of nucleic acid sequences which can have substitutions, deletions, or additions of one or more nucleotides, provided that the biological activity of the sequence is generally maintained. When relating to a protein sequence, the substituting amino acid generally has chemico-physical properties which are similar to that of the substituted amino acid. The similar chemico-physical properties include, similarities in charge, bulkiness, hydrophobicity, hydrophylicity and the like. The

term "functional derivatives" is intended to include "fragments", "segments", "variants", "analogs" or "chemical derivatives" of the subject matter of the present invention.

Thus, the term "variant" refers herein to a protein or nucleic acid molecule which is substantially similar in structure and biological activity to the protein or nucleic acid of the present invention.

The functional derivatives of the present invention can be synthesized chemically or produced through recombinant DNA technology. All these methods are well-known in the art.

As used herein, "chemical derivatives" is meant to cover additional chemical moieties not normally part of the subject matter of the invention. Such moieties could affect the physico-chemical characteristic of the derivative (e.g. solubility, absorption, half life, decrease of toxicity and the like). Such moieties are exemplified in Remington's Pharmaceutical Sciences (1980). Methods of coupling these chemical-physical moieties to a polypeptide or nucleic acid sequence are well-known in the art.

The term "allele" defines an alternative form of a gene which occupies a given locus on a chromosome.

As commonly known, a "mutation" is a detectable change in the genetic material which can be transmitted to a daughter cell. Aswell-known, a mutation can be, for example, a detectable change in one or more deoxyribonucleotide. For example, nucleotides can be added, deleted, substituted for, inverted, or transposed to a new position. Spontaneous mutations and experimentally induced mutations exist. A mutant polypeptide can be encoded from this mutant nucleic acid molecule.

As used herein, the term "purified" refers to a molecule having been separated from a cellular component. Thus, for example, a "purified protein" has been purified to a level not found in nature. A "substantially pure" molecule is a molecule that is lacking in most other cellular components.

A host cell or indicator cell has been "transfected" by exogenous or heterologous DNA (e.g. a DNA construct) when such DNA has been introduced inside the cell. The transfecting DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transfecting DNA may be maintained on a episomal element such as a plasmid.

BRIEF DESCRIPTION OF THE DRAWINGS

Having thus generally described the invention, reference will now be made to the accompanying drawings, showing by way of illustration a preferred embodiment thereof, and in which:

Figure 1 shows *A. niger* shuttle vectors constructed for this study. The various modules are indicated in the figure as follows, pUC18 sequences (pUC), glucoamylase transciption terminator (GlaTt), glucoamylase promoter region (GlaPr), ß-galactosidase coding region (LacA), the selectable marker (PyrG) and the sequence to support autonomous replication (AMA). Also indicated are the various unique restriction endonuclease sites that can be used to introduce or exchange modular elements in these vectors. Note: a translation termination codon and transcription termination region are present in the small *Aat*II to *Mlu*I fragment present in vectors pGla-GlaTt, pGlaPr-GlaTt-PyrG, pGlaPr-LacA-GlaTt-PyrG and pGlaPr-GlaTt-PyrG-AMA. Plasmids with the *LacA* coding region cloned into the *Nhe*I and *Fse*I generated backbones of pGla-GlaTt and pGlaPr-GlaTt-PyrG-AMA have also been constructed but are not shown in the figure; and

Figure 2 shows the various promoter regions used to express the *LacA* reporter in *A. niger*. pGlaPr-LacA-GlaTt-PyrG-AMA and several plasmids that are identical to it except for the regulatory sequences that direct *LacA* expression. Gla-483 through Gla-89 are GlaA promoter derivatives with varying amounts of the upstream information deleted. Gla-369 URS-1 through

Gla-369 URS-5 are various derivatives of Gla-369 with one to five copies of the URS_{GlaA} region between basepairs -369 and -483 inserted into its unique *MluI* site.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments with reference to the accompanying drawing which is exemplary and should not be interpreted as limiting the scope of the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention had at least three objectives. The first was to develop a modular set of cassettes and vectors for studying gene expression and protein secretion in filamentous fungi. The second was to develop a rational and efficient high-throughput reporter gene-based screen that could be used to isolate strains that express proteins of commercial importance at high levels. The third was to demonstrate that these expression vectors in combination with the high-throughput screen could be used to generate strains that express commercially valuable proteins at the high levels required for industrial applications.

Overview of the modular elements, expression cassettes and plasmid vectors of the present invention

The plasmid expression vectors constructed herein, pGlaPr-GlaTt, pGlaPr-LacA-GlaTt, pPkiPr-LacA-GlaTt, pGlaPr-LacA-GlaTt-PyrG and pGlaPr-LacA-GlaTt-PyrG-AMA, and pertinent features associated with several derivatives thereof are depicted in Figure 1 and Figure 2. pGlaPr-GlaTt is an integrating vector which can be used to express any protein coding sequence. It contains unique cloning sites for the insertion of protein coding information flanked by regulatory information for transcription initiation and transcription termination derived from the *A. niger GlaA* gene. The *GlaA* gene was used as the source of these control signals because it is quite well characterized (Hata et al. 1992; Verdoes et al., 1993; Fowler et al., 1990; Santerre et al., 1999) and

because it has been used to direct high levels of both homologous (Withers et al., 1998) and heterologous (Carrez et al., 1990; Jeenes et al., 1994; and Jeens et al 1993) protein expression. Analysis of the sequenced *GlaA* promoter region (Genbank accession # K02465) and its upstream sequences revealed a potential gene that coded for a protein with 42% identity to a *Candida rugosa* triacylglycerol lipase precursor (Genbank accession # AF044078). The start and stop codons for this putative lipase gene begin and end respectively 2840 and 660 base pairs upstream of the *GlaA* coding region. Thus *A. niger* expression vectors utilizing *GlaA* promoter regions extending beyond base pair -660 would include coding sequences for this lipase homolog. To avoid including this gene, only promoter sequences between bp -667 and -1 with respect to *GlaA* were included. This region includes, at its 5' end, the translational and transcriptional termination signals, and poly-adenylation site for the lipase gene followed by the entire *GlaA* promoter region.

pGlaPr-GlaTt-PyrG a selectable integrating plasmid was derived from pGlaPr-GlaTt by inserting a selectable marker *PyrG* (Oakley et al., 1987) module. pGlaPr-GlaTt-PyrG can be directly selected for and does not require cotransformation.

pGlaPr-GlaTt-PyrG-AMA was derived by inserting the AMA replicator sequence into pGlaPr-GlaTt-PyrG. This plasmid replicates autonomously in *A. niger.* It has unique cloning sites that can be used to place the coding sequence for any protein of interest under the regulation of *GlaA* control signals.

pGlaPr-LacA-GlaTt, pGlaPr-LacA-GlaTt-PyrG and pGlaPr-LacA-GlaTt-PyrG-AMA were derived from their parent plasmids pGlaPr-GlaTt, pGlaPr-GlaTt-PyrG and pGlaPr-GlaTt-PyrG-AMA by inserting the coding region of *LacA* into their backbones prepared by *Nhe*I and *Fse*I digestion. These three plasmids direct *LacA* expression with transcriptional control, initiation, polyadenylation and termination signals derived from the *GlaA* gene. pPkiPr-

LacA-GlaTt is identical to pGlaPr-LacA-GlaTt except that the *GlaA* promoter module has been replaced by the promoter region from another highly expressed gene, *PkiA* (de Graaff et al., 1992).

*Lac*A encodes a reporter suitable for studying both gene expression and protein secretion in A. niger

The *A. niger LacA* gene codes for a secreted ß-galactosidase (Kumar et al., 1992). To test its suitability as a reporter gene, the*LacA* coding region was cloned into the backbone of plasmid pGlaPr-GlaTt prepared by *Nhe*I and *Fse*I digestion. The resulting plasmid was designated pGlaPr-LacA-GlaTt. A second plasmid, pPkiPr-LacA-GlaTt, with *LacA* expressed from the *PkiA* promoter was also constructed. Lactase expression from both pGlaPr-LacA-GlaTt and pPkiPr-LacA-GlaTt was compared. Cotransformation was performed with 1 µg of plasmid pPyrG and 10 µg of pGlaPr-LacA-GlaTt or pPkiPr-LacA-GlaTt. Transformants harbouring both plasmids were obtained by screening for blue colonies on selective minimal medium indicator plates containing X-gal. Blue *PyrG*$^{+}$ colonies therefore identify transformants harbouring both integrating plasmids. To establish the levels of *LacA* expression 40 pGlaPr-LacA-GlaTt and 6 pPkiPr-LacA-GlaTt dark blue colonies were selected and grown in 30 ml liquid cultures and assayed for ß-galactosidase activity. The mean activity obtained for the pGlaPr-LacA-GlaTt and pPkiPr-LacA-GlaTt transformants was 4.3 (+/- 2.2) and 5.3 (+/- 3.1) units as compared to the less than 0.01 units expressed by transformants harbouring only pPyrG (Table 1). These results demonstrate that vectors pGlaPr-GlaTt and pPkiPr-GlaTt are capable of expressing the *LacA* reporter at high levels. They also demonstrate that *LacA* is a suitable reporter gene for fungal gene expression and protein secretion studies.

Table 1- *A. niger LacA* is a suitable reporter for monitoring secreted protein expression, and both *GlaA* and *PkiA* promoter modules can express the *LacA* reporter gene at high levels

Plasmid	*PyrG*$^+$ transformants per ug of PyrG containing plasmid	*PyrG*$^+$ and *LacA*$^+$ transformants[a]	ß-galactosidase activity as units per ml of culture medium[b]
PPyrG	279	0	<0.01
pPyrG and pGlaPr-LacA-GlaTt	376	286	4.3
pPyrG and pPkiPr-LacA-GlaTt	130	112	5.3
pGlaPr-LacA-GlaTt-PyrG	325	325	4.5

[a] Co-transformations were performed with 1 µg pPyrG and 10 µg of the indicated plasmids. pGlaPr-LacA-GlaTt-PyrG transformation was performed with 1 µg of plasmid DNA. Number of *PyrG*$^+$ *LacA*$^+$ transformants was determined by counting the number of blue *PyrG*$^+$ colonies present of minimal medium with X-gal.

[b] ß-galactosidase activity per ml of culture (where one unit of lactase hydrolizes 1 micromole of ONPG to ortho-nitrophenol per minute at 37C) was calculated as follows $(\Delta A_{410}/\text{min})(V_r)(D)/(V_e)(E_{410})$ where: $\Delta A_{410}/\text{min}$ = change in absorbance per minute, V_r = assay volume, D = dilution factor, V_e = enzyme volume and E_{410} = millimolar extinction coefficient of ortho-nitrophenol (3.5 $mM^{-1}cm^{-1}$). Note: The cultures used were 5 ml cultures of transformants that were selected for expressing relatively high levels of LacAp (dark blue colonies) as described in the Examples.

Transformation frequencies and reporter gene expression with a selectable integrating plasmid

The lactase producing transformants characterized above were obtained by cotransformation with plasmid pPyrG and either pGlaPr-LacA-GlaTt or pPkiPr-LacA-GlaTt. Cotransformation was required because the reporter gene expression cassettes were present on plasmids lacking a selectable marker. To simplify the transformation procedure and the characterization of transformants a vector that included a selectable marker was constructed. This plasmid, pGlaPr-LacA-GlaTt-PyrG, has the *E. nidulans PyrG* gene (prepared by PCR as described herein).

The frequencies of obtaining lactase producing transformants with the integrating plasmids pGlaPr-LacA-GlaTt and pGlaPr-LacA-GlaTt-PyrG were compared. Transformation frequency increased about 1.5-fold when the selectable marker and the *LacA* genes were both on the same plasmid (Table 1). The levels of ß-galactosidase expression directed by the GlaPr-LacA-GlaTt cassette were not significantly different (Table 1) regardless of whether LacAp was expressed from pGlaPr-LacA-GlaTt which required cotransformation with pPyrG, or from pGlaPr-LacA-GlaTt-PyrG which harbours its own selectable marker.

Autonomously replicating plasmids for genetic analysis of gene expression in *A. niger*

Traditional strains with improved protein expression characteristics have been isolated by multiple rounds of mutagenesis and screening using transformants that express the desired protein from an expression vector integrated into the genome. For this multiple rounds of mutagenesis followed by labour-intensive brute-force biochemical screening are typical. Furthermore, screening is typically time consuming and expensive. Although this approach has been successfully utilized to develop strains that express a number of commercially important proteins (Withers et al., 1998,

Carrez et al., 1990; Jeenes et al., 1994; and Jeenes et al 1993), strains developed using this approach cannot be easily adapted to express a different protein nor are they readily amenable to the identification and analysis of the genetic alterations that increase expression. Mutations that change gene expression can alter trans-acting factors or cis-acting sequences. For many genetic approaches aimed at the isolation and characterization of mutations that change gene expression it is important to be able to distinguish between trans- and cis-acting mutations.

To address these issues, a system that can be used for rapid high-throughput indicator plate screening that also facilitates the isolation and characterization of mutations that improve protein expression was developed. This system utilizes the AMA replicator sequence that supports autonomous replication. Adding a replicator to the expression vectors facilitates the identification of vector versus host mutations that alter reporter gene expression. An AMA based module was constructed and cloned into the unique *NotI* site of plasmid pGlaPr-GlaTt-PyrG. Reporter gene expression and the transformation frequency obtained with a *LacA* expressing derivative, pGlaPr-LacA-GlaTt-PyrG-AMA, were compared with the values obtained with the integrating plasmid pGlaPr-LacA-GlaTt-PyrG (Table 2). Levels of expression from both vectors were essentially the same; however, including the AMA replicator increased the transformation frequency 35-fold (Table 2) to 100-fold (data not shown).

Table 2- The AMA module supports, high transformation frequencies, autonomous replication and high levels of lactase expression

Plasmid	*PyrG*$^+$ transform-ants per µg of plasmid[a]	Proportion of total lactase activity in cell extracts	ß-galactosidase activity (secreted) as units per ml of culture medium
PGlaPr-LacA-GlaTt-PyrG	215	.05	4.3
PGlaPr-LacA-GlaTt-PyrG-AMA	7,075	.05	5.6

[a] Transformations and ß-galactosidase activity assays were determined as described for Table 1. The cultures used were 30 ml cultures of transformants that were selected for expressing relatively high levels of LacAp (dark blue colonies) as described in the Examples.

Of note, in accordance with a preferred embodiment of the present invention, the transformants obtained with the self-replicating vector could be cured of the plasmid and the plasmid could be retrieved into *E. coli* for further analysis. Plasmid retrieval was attempted with 9 independent pGlaPr-LacA-GlaTt-PyrG-AMA transformants. The data in Table 3 show that 8 of the 9transformants could be cured of their plasmids and that the plasmids could be readily retrieved into *E. coli.*

Table 3- pGlaPr-LacA-GlaTt-PyrG-AMA transformants of *A. niger* are unstable and the plasmid can be readily retrieved into *E. coli*

Plasmid	Plasmid retrieval into *E. coli*[a]	Plasmid stability [b]
pGlaPr-LacA-GlaTt-PyrG	< 1 per µg	1.00
pGlaPr-LacA-GlaTt-PyrG-AMA	2.7 X 10^3 per µg	0.03

[a]Plasmid DNA was retrieved into *E. coli* as described in the Examples. The frequency indicated is the number of transformants obtained by transforming strain DH5alpha with 1 µg of DNA isolated from the *A. niger* transformant indicated.

[b]Plasmid stability was determined by independently inoculating 9 transformants in 30 ml liquid cultures with uracil and uridine, following growth of the liquid cultures 0.05 grams (wet weight) of conidia were plated onto nonselective CM media plates and grown until a lawn of conidia appeared. After harvesting the conidia were plated (300 conidia per plate) onto nonselective MM plates with X-gal and 0.1% Triton. Stability values given represent the proportion of blue colonies (average for 8 of the 9 transformants) observed. One transformant was stable and did not produce any colourless colonies.

Autonomously replicating LacA reporter constructs can be used to characterize cis-acting regulatory information

It was of interest to demonstrate that the autonomously replicating plasmid system could be used to characterize cis-acting mutations. The utility of this system was demonstrated by characterizing a set of *GlaA* promoter deletions. For this a *GlaA* promoter deletion set was constructed (Fig.

2). The plasmids pGla-670Pr-LacA-GlaTt-PyrG-AMA through pGla-89Pr-LacA-GlaTt-PyrG-AMA were transformed into strain N593 and ß-galactosidase expression by the transformants in MM with maltose and in MM with glucose as the carbon source was determined. Two of the seven regions defined by these promoter deletions harbour upstream information important for the regulation of *GlaA* (Table 4). Sequentially deleting these two important upstream regions, overlapping sequences between -483 and -454 bp, and -454 and -369 bp, reduced expression from 2.5 units to 0.7 units and less than 0.01 units respectively. The autonomously replicating vector pGlaPr-LacA-GlaTt-PyrG-AMA and its derivatives can therefore be used to characterize plasmid-based sequences that affect expression levels and/or the efficiency of protein secretion.

Table 4- GlaA promoter deletion analysis

Plasmid[a]	*PyrG*$^+$ transformants per µg pPyrG[b]	ß-galactosidase activity as units per ml of culture medium[c]
pGla-670Pr-LacA-PyrG-AMA	1,000	1.5 (+/- 1.1)
pGla-483Pr-LacA-PyrG-AMA	22	2.5 (+/- 1.5)
pGla-454Pr-LacA-PyrG-AMA	715	0.7 (+/- 0.28)
pGla-369Pr-LacA-PyrG-AMA	172	< 0.01
pGla-253Pr-LacA-PyrG-AMA	770	< 0.01
pGla-145Pr-LacA-PyrG-AMA	639	< 0.01
pGla-89Pr-LacA-PyrG-AMA	248	< 0.01

[a] The plasmids indicated are identical except they harbour varying portions of the *GlaA promoter.* Each promoter construct extends from the identical 3' nucleotide (-1 relative to the start codon) to the 5' nucleotide indicated in the plasmid designations.

[b] Transformations were performed using 4 X 10^6 frozen competent cells rather than the method described in the Examples.

[c] Activity per ml of medium, transformants were grown in 5 ml culture. Note: Expression levels given are averages for 10 randomly selected *PyrG*$^+$ transformants.

Isolation of *A. niger* mutants with increased expression of secreted LacAp

The modular expression system of the present invention was tested to assess whether it could be used for high-throughput strain improvement screening to obtain mutants that secreted much higher amounts of ß-galactosidase than the 1.5 units per ml typically expressed by pGlaPr-LacA-GlaTt-PyrG-AMA transformants. For this a pGlaPr-LacA-GlaTt-PyrG-AMA transformant was subjected to three rounds of mutagenesis where each round was followed by high-throughput screening for mutants that expressed increased levels of lactase. Each round of mutagenesis was performed as follows. Conidia were mutagenized with nitrosoguanidine (15 µg/ml for 40 minutes) to give a final survival of 0.2%. Following mutagenesis the conidia were plated on minimal medium plates with 0.1% Triton 100 and X-gal. For each round, 10,000 survivors were screened for mutants that produced significantly higher levels of lactase (dark blue colonies). Sixty of the highest lactase producing colonies were isolated and grown in 5 ml MM medium cultures. Following growth for 6 days levels of lactase in the medium were determined. The five highest producing isolates were then grown in 30 ml cultures and assayed for lactase production. The three isolates that produced lactase at the highest levels were subject to another round of mutagenesis and high-throughput screening. After three rounds of screening 27 isolates were obtained (Table 5). Lactase expression by these 27 isolates was increased 5- to 46-fold during the three rounds of high-throughput screening. The isolate that produced the highest levels of lactase, isolate 31-pGlaPr-LacA-GlaTt-PyrG-AMA-12-4-24, expressed 73 units/ml or 1.1 mg/ml of secreted lactase.

Table 5- Mutants isolated by high-throughput screening

Round of high-throughput screening[a]	Number of mutagenized clones screened on indicator plates	Number of high-producing clones assayed using liquid cultures	Units of lactase expression (average)[b]
original transformant	NA	NA	1.9
round one	10,000	60	8.0
round two	30,000	180	15
round three	90,000	540	27

[a] *A. niger* transformed with plasmid pGlaPr-LacA-PyrG-AMA was subjected to four rounds of mutagenesis followed by screening for high lactase production on minimal medium X-gal plates. Mutagenesis was performed with nitrosoguanidine as described in the Materials and Methods. 1 X 10^4 viable conidia were screened for each mutagenesis.

[b] From each mutagenesis the 60 highest LacAp producers, identified by screening on X-gal indicator plates, were selected for further analysis using 5 ml liquid cultures. Lactase expression levels given are the averages obtained with the isolates from each mutagenesis (3 from the first round, 9 from the second round and 27 from the third round) that produced the highest levels ofLacAp in liquid cultures.

Lactase is widely used in the milk industry, both for the hydrolysis of lactose in milk products prepared for people with lactose intolerance and for treating milk industry byproducts such as the whey produced during the manufacture of cheese. These results demonstrate that the reporter gene system and high-throughput screen of the present invention can be used to generate and isolate strains with dramatically improved yields of a commercially valuable secreted protein. It will be recognized that the reporter gene system and high-throughput screen of the present invention can be adapted to numerous utilities.

Mutational analysis of cis-acting GlaA information

Deletion analysis revealed that upstream deletions extending beyond bp-483 significantly reduced promoter activity. Deleting to bp -454

reduced expression 3.5-fold relative to expression directed by the full length promoter. Deleting the information upstream of bp -369 abolished promoter activity. Furthermore, since the TATA element is located atbp -117, apparently neither the information where the basal transcription apparatus assembled nor the transcription initiation region were missing from the pGla-369Pr-LacA-GlaTt-Pyr-AMA construct. Thus pGla-369Pr-LacA-GlaTt-Pyr-AMA is unable to express lactase because critical regulatory information had been deleted. Levels of gene expression can often be increased by increasing the copy number of an upstream regulatory element. To test this the region between nucleotides -369 and -483 of the *GlaA* region was amplified by PCR using oligonucleotides PGLA5'-483 and PGLA3'URR-369, and plasmid pGlaPr-LacA-GlaTt-PyrG as template. The resulting product was cleaned by ethanol precipitation, digested with *Mlu*I and cloned into the backbone of plasmid pGLA-369 (Fig. 2). Isolates harbouring 1 to 5 copies of the region were identified and transformed into *A. niger.* Although including a single copy of this region increased expression to relatively high levels, expression did not increased significantly with additional copies in a fashion consistent with cooperative binding of a critical transcriptional activator (Table 6). These results suggest that *A. niger* expresses relatively low levels of a factor that is essential for *GlaA* expression, and that excess copies of the DNA element for this protein can titrate out its activity. Furthermore, since pGlaPr-369-LacA-GlaTt-PyrG-AMA is an autonomously replicating plasmid with *GlaA* promoter sequences devoid of upstream regulatory information, it can be used to clone and characterize cis-acting upstream regulatory information. It will be recognized that the sequences of the DNA element for *GlaA* could be used to clone the factor which appears essential for the expression of *GlaA*. Methods for cloning proteins which bind to nucleic acid sequences are well-known in the art.

Table 6- The *GlaA* region from -483 to -369 includes upstream regulatory sequences that support the activation of transcription

Number of copies of the *LacA* URR[a] cloned into pGla-369Pr-LacA-GlaTt-PyrG-AMA	Lactase expression
0	0.08 units
1	1.8 units
2	1.5 units
3	1.6 units
4	1.8 units
5	2.2 units

[a]The *GlaA* region between nucleotides -483 and -369 was amplified by PCR as described in the Materials and Methods. The resulting PCR product was cloned in various copy numbers into the unique *Mlu*I site of pGla-369Pr-LacA-GlaTt-PyrG-AMA.

Once it had been established that this region (bp -483 to -369) included upstream regulatory sequences (URS) important for *GlaA* expression, a PCR-based strategy was used to generate a library of randomly mutated $GlaA_{URS}$ sequences. A library of mutated elements, cloned into the unique *Mlu*I site of plasmid pGla-369Pr-LacA-GlaTt-PyrG-AMA were screened using the high-throughput indicator plate screen. A total of 33 mutant URSs that directed significantly increased levels of expression and 72 mutants that directed significantly reduced levels of expression were identified and retrieved into *E. coli* for further analysis. Initially these mutant elements will be subject to DNA sequencing to identify residues critical for transcriptional regulation by the $GlaA_{URS}$ region.

Thus, the present invention provides the means to modulate the activity of the promoter and/or the expression levels of proteins operably linked to such promoters.

Lactase expression with the GlaPr-LacA-GlaTt gene replacement cassette

When engineering strains for the commercial production of proteins, it is preferable to minimize changes to the host strain since these can complicate the process leading to regulatory approval. Although the expression vectors and screening methods outlined above facilitate rational strain improvement strategies, a gene replacement cassette will be employed to express specific products. In one particular embodiment, this cassette consists of three elements. First, it includes a derivative of the *GlaA* promoter region that directs expression at levels that are nine-fold higher than levels obtained with the original wild-type promoter. The improvements engineered into the promoter module are based on mutations introduced into the autonomously replicating vectors obtained during high-throughput screening (see above sections). The second element is the lactase-encoding portion of *LacA*. It encodes a commercially valuable product but can easily be replaced by any other gene. Finally the cassette includes 1.1 kbp derived from genomic sequences immediately downstream of the *GlaA* coding region. It includes the information for transcription termination and polyadenylation.

This GlaPr-LacA-GlaTt cassette can be used to replace the wild type *GlaA* locus. The utility of this system for engineering strains is evident in the levels of lactase expression obtained using this cassette (Table 7), since expression of lactase in amounts of 1 gram per liter make the resulting strain suitable for the production of lactase for the milk fluids industry and the treatment of lactose intolerance.

Table 7- Lactase expression by transformants obtained with the GlaPr-LacA-GlaATt gene replacement expression cassette

Number of GlaPr-LacA-GlaATt copies/genome	Lactase expression
0	0.01 units
1	1.0 units
2	4.0 units
3	6.0 units
4	4.3 units
5	15 units

[a]Expression levels are for the transformant (identified by Southern analysis) that gave the highest level of expression.

LacAp fusion proteins for heterologous protein expression

It is often difficult to express heterologous proteins efficiently in *A. niger.* To effect efficient expression it is often necessary to subject transformants expressing modest levels of the desired protein to several rounds of mutation followed by brute-force screening. In view of overcoming these drawbacks, an autonomously replicating vector that can be used to express heterologous proteins as *LacA* fusions has been developed. In one particular embodiment, the system provides two advantages over existing systems for engineering strains that express heterologous proteins at high levels. First the heterologous protein does not require its own signal sequences for secretion, because ß-galactosidase, an efficiently secreted protein, provides this information. Second, since ß-galactosidase is not inactivated by fusing protein sequences to its carboxyl end, simple end-point or indicator plate assays performed to determine levels of ß-galactosidase expression also estimate expression levels of the protein fused to its carboxyl terminus. Vectors expressing a protein of interest as a fusion with ß-galactosidase can therefore be subjected

to high-throughput screening to create mutants that express the heterologous protein at high levels.

The present invention is illustrated in further detail by the following non-limiting examples.

EXAMPLE 1

Strains and growth conditions

Strain N814 (cspA1;fwnA1;pyrG5;nicB5) and N593 (cspA1;pyrG6) were provided by C.J. Bos (Wageningen Agricultural University), and strain A742 was obtained from the Fungal Genetics Stock Centre (FGSC). All the above strains are derived from strain N402, a UV induced *cspA*1 mutant derived from strain ATTC 9029 (Bos et al, 1988). The strains used are listed in Table 8. Unless otherwise stated all liquid cultures of *A. niger* used for protein expression studies were grown at 30°C and 200 rpm in an air incubator shaker for six days. Agar media plates were incubated at 30°C. Minimal media (MM) as described previously (Kafer et al., 1977) with 15% carbohydrate was used for liquid cultures. Agar (final concentration of 1.5%) was added for plates. When using strain N814, nicotinic acid was added (1 µg/ml final concentration). Ampicillin (100 µg/ml of culture) to prevent bacterial growth was added to all 30-ml cultures. Complete medium (CM) (Kafer et al., 1977) was used when growing strains for protoplast generation as well as for the isolation of genomic DNA . *E. coli* strain DH5alpha (Woodcock et al., 1989) was used for the maintenance and production of plasmid DNAs.

Table 8- Strains used in this study

Strains	Genotype	Source
A742	*cspA*1;*pyrG*5	FGSC
N814	*cspA*1;*fwnA*1;*pyrG*5;*nicB*5	C.J. Bos
N593	*cspA*1;*pyrG*6	C.J. Bos
DH5alpha	*supE*44 Δ*lac*U169 (Ø80 *lacZ*ΔM15)*hsdR*17 *recA*1 *endA*1 *gyrA*96 thi-1 *relA*1	D.M. Woodcock

EXAMPLE 2

Enzyme assays

Six day cultures were filtered through Miracloth™ and the media were kept on ice. ß-galactosidase assays were initiated by adding 2µl of media to 100µl of reaction buffer, 50mM sodium acetate pH 4.1, 2 mg/ml ofortho-nitrophenyl-ß-D-galactoside (ONPG) and incubated for 10 minutes at 37C. Reactions were stopped by adding 900µl of 0.1M sodium carbonate. Units of ß-galactosidase activity per ml of culture (where one unit of lactase hydrolizes 1 millimole of ONPG to ortho-nitrophenol per minute at 37C) were calculated as follows $(\Delta A_{410}/min)(V_r)(D)/(V_e)(E_{410})$ where: ΔA_{410}/min = change in absorbance per minute, V_r = assay volume, D = dilution factor, V_e = enzyme volume and E_{410} = µmolar extinction coefficient of ortho-nitrophenol ($3.5 \times 10^3\ ^{\mu}M^{-1}cm^{-1}$).

EXAMPLE 3

Isolation and manipulation of DNA

In vitro DNA manipulations, *E. coli* transformations and the isolation of plasmid DNAs from *E. coli* were performed as described previously (Sambrook et al., 1989, and Williams et al. 1996). Genomic DNA was isolated

using a modification of the method of E. Kafer (Oza and Kafer, 1990). *A. niger* cultures were grown overnight in CM at 30°C and 150 rpm. Mycelia were harvested by passing the culture through Miracloth, rinsed with distilled water and blotted dry. The mycelial mass was then placed in a mortar containing liquid nitrogen and ground to a fine powder with a pestle. The powdered extract was then added to a solution of 10mM TrisHCl, 100mM EDTA pH 8.0 (1 g mycelial wet weight/5ml). Next 20% Sarkosyl (0.5ml/1g mycelial wet weight) followed by 0.2ml of RNaseA (10mg/ml in 50mM sodium acetate) were added. After incubation at 65°C for 30 minutes, the extract was centrifuged at room temperature for 30 minutes at 13,000 rpm in a Beckman JA17 centrifuge rotor. The supernatant, after transfer to a new centrifuge tube, was extracted two times with an equal volume of phenol:chloroform and once with chloroform before DNA precipitation with 0.6 volumes of isopropanol. The precipitated DNA was pelleted by centrifugation in a Beckman JA17 rotor at 15,000 rpm for 30 minutes, rinsed with 70% ethanol, and resuspended in water. PCR amplifications using *A. niger* genomic DNA templates were performed using a Perkin Elmer GeneAmp 2500 thermal cycler™. The oligonucleotides used are presented with their pertinent features in Table 9.

Table 9 - Oligonucleotides used in this study

Oligo designation	Oligo description	Oligo sequence[a]
PGlaPr5'	GlaA promoter sense primer the *Not*I and *Aat*II sites are underlined, homology from -667 to-649	5'-AGAATGCGGCCGCATGACGTC**CCACATCTGATGCCATTGG**-3'
PGlaPr3'	GlaA promoter antisense primer, the *Nhe*I and *Xba*I sites are underlined, mutations included in the oligo are italicized, homology from -24 to -1	5'-AGAATGGCCGGCCATTCTAGA**TGCT***AGC***GTGTAATGATGCTGGGG**-3'
*FSE*I LINKER		5'-TGGCCGGCCA-3'
PPyr5'	PyrG sense primer, a *Not*I site is underlined, homology from -476 to -459	5'-ATAAGAATGCGGCCGC**GCAACTTCCTCGAGAACG**-3'
PPyr3'	E. nidulans PPyrG sense primer, an *Aat*II site is underlined, homology from -903 to -921	5'-AGAATGACGTC**CCCTTTTAGTCAATACCG**-3'
PPki5'	E. nidulans PyrG antisense primer , an *Mlu*I site is underlined, homology from -829 to -811	5'-TTGTCACTCTACGCGT**GTTCTTGCGAAGGGCATTG**-3'

Table 9 (continued)

Oligo designation	Oligo description	Oligo sequence[a]
PPki3'	A. niger PkiA sense primer, the *Nhe*I and *Xba*I sites are underlined, homology from -20 to -1	5'-GGATTCTAGAGCTAGCT **CTTGACGGATGATTGATCTC**-3'
*Hin*dIII/*Not*I-top	*Hin*dIII adaptor in italics with the NotI site underlined	5'-*AGCTT*GCGGCCGCTAA-3'
*Hin*dIII/*Not*I-bottom	*Hin*dIII adaptor with the *Not*I site underlined	5'- TTAGCGGCCGCA-3'
PGLA5'-483	GlaA sense primer, an *Mlu*I site is underlined, homology to -483 to -464	5'ATAACGCGT**TTATACCAGCC AATCAAGTC**-3'
PGLA5'-454	GlaA sense primer, an *Mlu*I site is underlined, homology to -454 to -435	5'-ATAACGCGT**GACCGGGGAC GGCGAATGCC**-3'
PGLA5'-369	GlaA sense primer, an *Mlu*I site is underlined, homology to -369 to-35	5'-ATAACGCGT**GCACAGGGC CATTCTGCAGC**-3'
PGLA5'-253	GlaA sense primer, an *Mlu*I site is underlined, homology to -253 to -234	5'-ATAACGCGT**CCGTTCACAA GCTGAAGAGC**-3'
PGLA5'-145	GlaA sense primer, an *Mlu*I site is underlined, homology to -145 to -126	5'-ATAACGCGT**TCAGCTTCCCC TCGTGCAGA**-3'

Table 9 (continued)

Oligo designation	Oligo description	Oligo sequence[a]
PglaTt5'	GlaA terminator region sense primer, the *Eco*RI and *Fse*I sites are underlined, homology to +2136 to 2155	5'-CGGAATTCTTTGGCCGGCC**CGA CCGCGACGGTGACTGAC**-3'
PGlaTt3'	GlaA terminator region antisense primer, an *Ssp*I site is underlined, homology to +3179 to +3198	5'-CGCGAATATT**CCGGAGATCCTGATC ATCCG-3'**
PLac5'	LacA sense primer, an *Nhe*I site is underlined, homology to -3 to +17	5'-AGAATGCTAGC**ATCATGAAGC TTTCCTCCGC-3'**
PLac3'	LacA antisense primer, an *Fse*I site is underlined, homology to +3530 to +3510	5'TCTTAGGCCGGCC**AACTTCTAA GCTCCTCCGGAC**-3'

[a]Boldface letters represent nucleotides identical to coding or noncoding strand nucleotides (unless designated otherwise in the oligo descriptions). For example boldface letters for PGla5' (-667 to -649) are identical to *GlaA* residues -667 to -649 relative to the first nucleotide of the coding region.

EXAMPLE 4

Transformation of *A. niger*

Protoplasts of *A. niger* were prepared essentially as described previously (Debets and Bos, 1986). Complete medium was inoculated with conidia of *A.niger* at a concentration of 10^6 conidia/ml, and grown overnight (16 hours) at 30°C and 150 rpm in a New Brunswick model G76 waterbath shaker. Mycelia (10 g wet weight), harvested by filtration through Miracloth (Calbiochem) and rinsed with distilled water, were added to 20 ml of lytic solution (0.7M sodium chloride, 0.2M calcium chloride pH 5.8, 10mg/ml Novozyme 234) . Digestion was allowed to proceed for 1-2 hours at 30°C, with shaking at 150rpm. The final yield of protoplasts varied between 1 X 10^7 and 4 x 10^7 protoplasts per gram of mycelia. Protoplasts were harvested by centrifugation in a clinical centrifuge at 2,800 rpm. Three washes were carried out, one by suspension in 0.7 M KCl and two by suspension in 1M sorbitol/50mM calcium chloride (S/C). Each wash was followed by harvesting at 2,800 rpm in a clinical centrifuge at room temperature. After the final wash the protoplasts were suspended in S/C (1 x 10^8 protoplasts/ml) and used for transformations performed essentially as described previously (Werner et al., 1987). The protoplasts (200µl containing 2 x 10^7 cells) were incubated on ice with 1-10µg of transforming DNA (maximum volume 10µl) and 50µl of PEG solution (25% polyethyleneglycol 8000 in 50mM $CaCl_2$, 10mM Tris-HCl pH 7.5), then 2 ml of PEG solution and 4 ml of S/C were added. The transformed protoplasts, harvested by centrifugation at 3,000 rpm for 5 minutes and resuspended in 500µl of S/C, were plated using agar overlays. For transformations with plasmids expressing *A. niger* ß-galactosidase MM plates containing 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (Xgal) at a final concentration of 40µg/ml were used.

EXAMPLE 5

Construction of modular DNA elements, expression cassettes and plasmid vectors

The 1057 bp *GlaA* transcription termination and polyA signal module (GlaTt) was generated by PCR amplification using *A. niger* genomic DNA template and oligonucleotides PGlaTt5' (complementary to nucleotides +2136 to +2155 where numbering throughout is relative to the first nucleotide of the relevant start codon unless stated otherwise) and PGlaTt3' (complementary to nucleotides +3179 to +3198). In addition PGlaTt5' has an 19 nucleotide extension that includes *Eco*RI and *Fse*I sites and PGlaTt3' had an 10 nucleotide 5' extension that includes an *Ssp*I site.

The promoter region module (GlaPr), which includes bp -667 to bp -1 of the *GlaA* gene region, was generated by PCR amplification using *A. niger* genomic DNA and oligonucleotides PGlaPr5' (complementary to nucleotides -667 to -649) and PGlaPr3'. PGlaPr3' is complementary to the *GlaA* coding strand from bp -1 to -24 except for the replacement of residues -7 to -5 (CTC) with GCT. These changes introduce an *Nhe*I site extending from bp -7 to -2 into the PCR product. PGlaPr3' has a 5' extension that includes *Fse*I and *Xba*I sites. PGlaPr5' has a 5' extension that includes *Not*I and *Aat*II sites.

The GlaTt and GlaPr modules were sequentially cloned into pUC18 as follows. First the PCR-generated GlaTt region was digested with *Eco*RI and *Ssp*I and cloned into a pUC18 backbone prepared by *Eco*RI and *Ssp*I digestion. The resulting intermediate plasmid, pGlaTt, was sequentially digested with *Sap*I, end-filled with Klenow polymerase and digested with *Fse*I. The PCR-generated promoter module (GlaPr) after *Fse*I digestion was inserted into the pGlaTt backbone to generate pGlaPr-GlaTt.

pGlaPr-GlaTt-PyrG (Figure 1), an integrating plasmid with the *A. nidulans PyrG* gene as a selectable marker, was constructed as follows.

Primers PPyr5' (complementary to nucleotides -476 to -459 of the noncoding strand) and PPyr3' (complementary to nucleotides +903 to +921 of the coding strand) were used to PCR amplify *PyrG* using plamid pPyrG as template. The resulting PCR product, which has *Not*I and *Aat*II sites at its 5' and 3' termini, was digested with *Not*I and *Aat*II and cloned into the backbone of pGlaPr-GlaTt prepared by *Not*I and *Aat*II digestion.

Autonomously replicating plasmid pGlaPr-GlaTt-PyrG-AMA was constructed by cloning most of the previously described AMA replicator sequence (Gems et al., 1991) into pGlaPr-GlaTt-PyrG that had been linearized by *Not*I digestion. The full length AMA sequence as originally isolated is 5991 bp (Gems et al., 1991); however, the minimal size known to support efficient autonomous replication is 5259 bp (Aleksenko et al., 1996). This slightly smaller AMA sequence was gel purified following digestion of plasmid pUCAMA with *Hin*dIII. The isolated fragment was ligated to a *Hin*dIII/*Not*I adaptor generated by annealing two oligos (*Hin*dIII/*Not*I-1 and *Hin*dIII/*Not*I-2) that had been phosphorylated using polynucleotide kinase. After digestion with *Not*I the linker-AMA product was inserted into the unique *Not*I site of plasmid pGlaPr-GlaTt-PyrG.

The *LacA* reporter module was prepared by PCR amplification using primers PLacA5' and PLacA3' and *A. niger* genomic DNA. The resulting PCR product contains *LacA* sequences from base pair -13 to +100 relative to the start and stop translation codons respectively. In addition to *LacA* sequences the PCR product includes *Nhe*I and *Fse*I sites (encoded in the two PCR oligonucleotides) at its 5' and 3' ends respectively. The resulting *LacA* fragment was digested with *Fse*I and *Nhe*I and cloned into the backbones of pGlaPr-GlaTt, pGlaPr-GlaTt-PyrG and pGlaPr-GlaTt-PyrG-AMA generated by *Fse*I and *Nhe*I digestion. The resulting integrating and autonomously replicating plasmids designated pGlaPr-LacA-GlaTt, pGlaPr-LacA-GlaTt-PyrG and pGlaPr-LacA-

GlaPr-PyrG-AMA respectively direct *LacA* expression using transcriptional regulation, initiation and termination signals derived from the *GlaA* gene.

EXAMPLE 6

*Gla*A promoter deletion set

The six *GlaA* promoter region derivatives depicted in Figure 2 were prepared and inserted into the backbone of pGlaPr-LacA-GlaTt-PyrG-AMA as follows. The DNA for each promoter deletion derivative was prepared by PCR amplification using vector pGlaPr-LacA-GlaTt-PyrG as template. The downstream or 3' oligonucleotide used to generate the six promoter regions was PGlaPr3', the same oligonucleotide used to prepare the *GlaA* promoter region present in construct pGlaPr-GlaTt. In combination with the downstream oligo six upstream oligos were used, PGla5'-483, PGla5'-454, PGla5'-369, PGla5'-253, PGla5'-145 and PGla5'-89. Beginning at their 5' ends each of these oligonucleotides contained three filler nucleotides, followed by an *Mlu*I site then a 20 base 3' end identical to various portions of the *GlaA* coding strand. The most upstream nucleotide homologous to *GlaA* promoter region sequences is indicated in the oligo designations. The six PCR products were individually digested with *Mlu*I and *Nhe*I and cloned into the large fragment of pGlaPr-LacA-GlaTt-PyrG-AMA prepared by *Mlu*I and *Nhe*I digestion, phosphatase treatment and isolation following agarose gel fractionation.

EXAMPLE 7

Cloning the PkiA promoter

The 829 base-pair (bp) *PkiA* promoter fragment was prepared by PCR-amplification from *A. niger* genomic DNA using oligos PPki5' and PPki3'. Proceeding from its 3' end PPki5' has 20 nucleotides that anneal to nucleotides -811 to -829 of the *PkiA* region, then a six nucleotide *Mlu*I site and finally a 10 nucleotide filler region at its 5' end. PPki3' has 21 nucleotides matching bases -1

to -21 of the noncoding strand at its 3' end followed by 12 nucleotides encoding adjacent *Nhe*I and *Xba*I sites and finally four filler nucleotides. The PCR-generated *PkiA* promoter fragment was cloned into the backbone of pGlaPr-LacA-GlaTt-PyrG-AMA prepared as described for the *GlaA* promoter deletion set.

It will be recognized that other fusion proteins could be designed in accordance with the present invention. For example, fusions comprising domains enabling affinity purification (or otherwise) linked to the protein sequence of interest by a protease site, as commonly known, could be used.

Non-limiting examples of such fusion proteins include hemaglutinin fusions and Gluthione-S-transferase (GST) fusions and Maltose binding protein (MBP) fusions. In certain embodiments, it might be beneficial to introduce a protease cleavage site between the two polypeptide sequences which have been fused. Such protease cleavage sites between two heterologously fused polypeptides are well-known in the art.

Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

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WHAT IS CLAIMED IS:

1. An expression vector capable of enabling a systematic analysis of gene expression and/or high-throughput strain improvement screens in a filamentous fungi comprising:

a) a selectable marker for said filamentous fungi; and

b) a promoter operably linked to a nucleic acid sequence encoding a protein of interest or part thereof.

2. The expression vector of claim 1, based on a pUC vector backbone.

3. The expression vector of claim 1 or 2, further comprising an assayable reporter gene operably linked to said promoter, enabling the quantification of the expression level of said protein of interest and/or of the activity of said promoter.

4. The expression vector of claim 1, 2 or 3, wherein said selectable marker is PyrG and said promoter is the glucoamilase promoter.

5. The expression vector of claim 1, 2, 3 or 4, wherein said protein of interest is LacA and said filamentous fungi is *A. niger*.

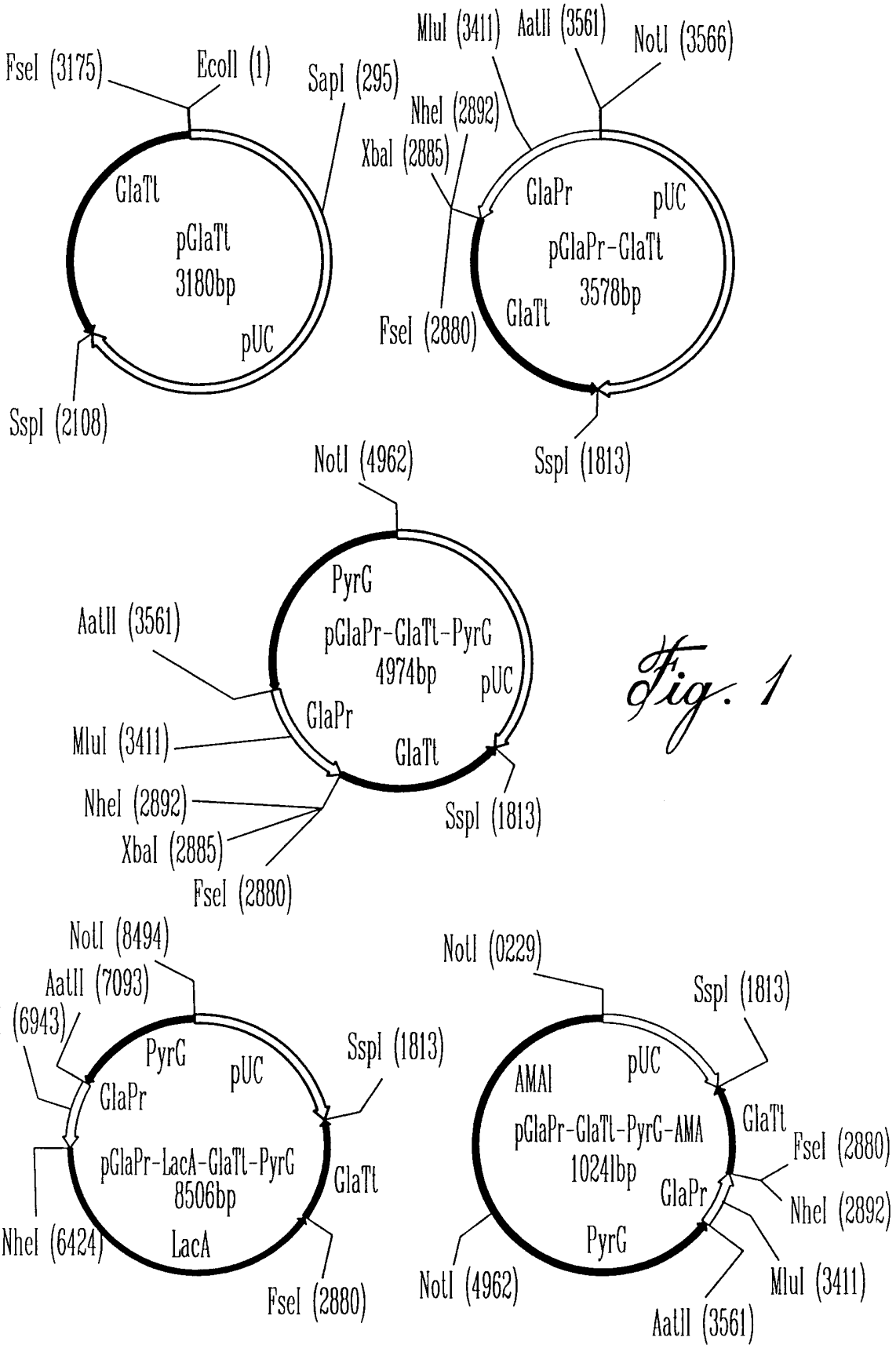
6. The expression vector of claim 1, 2, 3, 4 or 5, wherein the expression of said protein of interest is increased by about 100 times, and preferably by about 1000 times, as compared to the level of expression thereof in a control filamentous fungi strain.

7. A filamentous fungi strain, enabling the expression of a protein of interest to greater than about 100 times, and preferably greater than about

1000 times, as compared to the level thereof in parent strain of said filamentous fungi strain.

8. A method for isolating a filamentous fungi strain that expresses a protein of interest at high levels, comprising a use of a vector in accordance with the present invention.

1/2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EcoII (1)
SapI (295)
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pGlaTt
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pUC
SspI (2108)
MluI (3411)
AatII (3561)
NotI (3566)
NheI (2892)
XbaI (2885)
GlaPr
pUC
pGlaPr-GlaTt
3578bp
GlaTt
FseI (2880)
SspI (1813)
NotI (4962)
PyrG
pGlaPr-GlaTt-PyrG
4974bp
pUC
AatII (3561)
GlaPr
MluI (3411)
GlaTt
NheI (2892)
SspI (1813)
XbaI (2885)
FseI (2880)
NotI (8494)
AatII (7093)
MluI (6943)
PyrG
pUC
SspI (1813)
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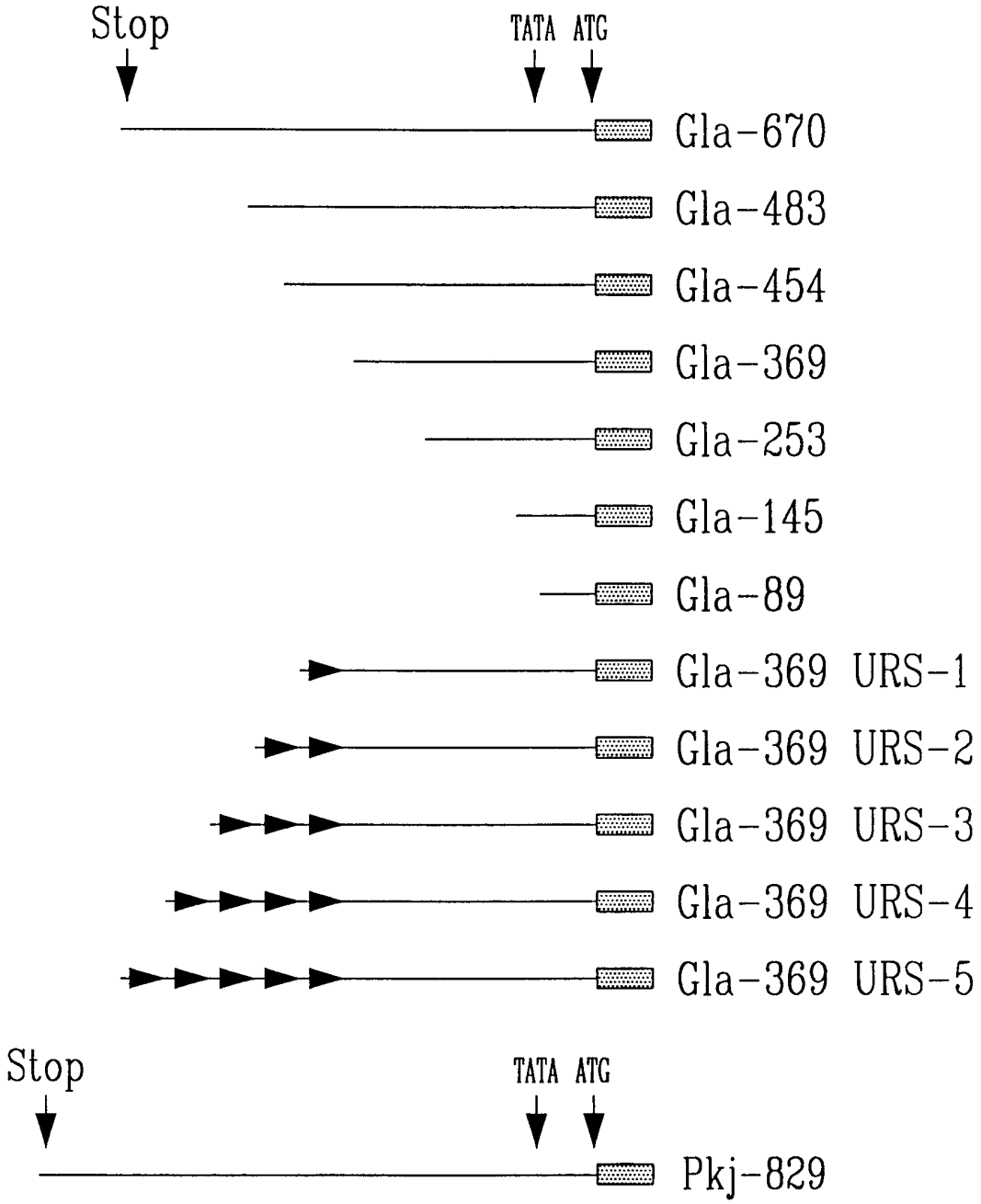
GlaTt
NheI (6424)
LacA
FseI (2880)
NotI (0229)
SspI (1813)
AMAI
pUC
pGlaPr-GlaTt-PyrG-AMA
10241bp
GlaTt
FseI (2880)
GlaPr
NheI (2892)
PyrG
NotI (4962)
MluI (3411)
AatII (3561)

Fig. 1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ATG
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Gla-483
Gla-454
Gla-369
Gla-253
Gla-145
Gla-89
Gla-369 URS-1
Gla-369 URS-2
Gla-369 URS-3
Gla-369 URS-4
Gla-369 URS-5
Stop
TATA
ATG
Pkj-829

Fig. 2

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/CA 00/01084

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/80 C12N15/67 C12N1/15

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 625 577 A (GENENCOR INTERNATIONAL, INC.) 23 November 1994 (1994-11-23) page 3, line 35 - line 54 page 5, line 1 - line 15; examples 1,7 ---	1-8
X	EP 0 225 078 A (PANLABS, INC.) 10 June 1987 (1987-06-10) page 2, line 50 -page 4, line 56 ---	1-4,6-8
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 January 2001	25/01/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montero Lopez, B

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INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/CA 00/01084

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	YOLANDA M.J.T. DE RUITER-JACOBS ET AL.: "A gene transfer system based on the homologous pyrG gene and efficient expression of bacterial genes in Aspergillus oryzae" CURRENT GENETICS, vol. 16, 1989, pages 159-163, XP000971454 the whole document ---	1-8
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X	WO 89 01969 A (NOVO INDUSTRI A/S) 9 March 1989 (1989-03-09) page 2, line 32 -page 4, line 10 page 5, line 17 - line 35 page 11, line 34 -page 12, line 13; example 5 -----	1-4,6-8

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: partially 7

Present claim 7 relates to a product defined by reference to a desirable characteristic or property, namely a fungus enabling the expression of a protein to greater than 100 times as compared to the level of a parent strain. The claim covers all strains having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such strains. In the present case, the claim so lacks support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claim also lacks clarity (Article 6 PCT). An attempt is made to define the product by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claim which appear to be clear, supported and disclosed, namely those parts relating to the strains disclosed in tables 1-7.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No
PCT/CA 00/01084

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