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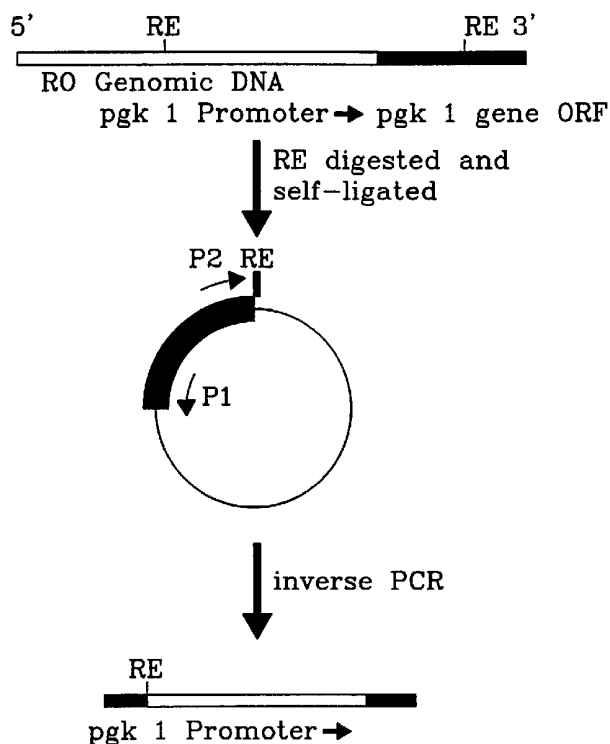
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[Continued on next page]

(54) Title: A PROMOTER SEQUENCE OF 3-PHOSPHOGLYCERATE KINASE GENE 1 OF LACTIC ACID-PRODUCING FUNGUS *RHIZOPUS ORYZAE* AND A METHOD OF EXPRESSING A GENE OF INTEREST IN FUNGAL SPECIES



(57) Abstract: The present invention provides the promoter clone discovery of phosphoglycerate kinase gene 1 of a lactic acid-producing filamentous fungal strain *Rhizopus oryzae*. The isolated promoter can constitutively regulate gene expression under various carbohydrate conditions. In addition, the present invention also provides a design of an integration vector for the transformation of a foreign gene in *Rhizopus oryzae*.



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**A PROMOTER SEQUENCE OF 3-PHOSPHOGLYCERATE KINASE GENE 1  
OF LACTIC ACID-PRODUCING FUNGUS *RHIZOPUS ORYZAE* AND A  
METHOD OF EXPRESSING A GENE OF INTEREST IN FUNGAL SPECIES**

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**FIELD OF THE INVENTION**

The present invention is a promoter sequence of 3-phosphoglycerate  
kinase gene 1 of lactic acid-producing fungus *Rhizopus oryzae* and a method of  
expressing a gene of interest in fungal species.

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**BACKGROUND OF THE INVENTION**

The genus of *Rhizopus* is versatile in the production of biocatalysts such  
as glucoamylase and lipase and chemicals including L-(+)-lactic acid, fumaric  
acid, and ethanol. *Rhizopus* is the member of the order Mucorales, which is  
within the class *Zygomycetes* of the division *Amastigomycota*. *Rhizopus oryzae*  
(ATCC 9363) is the best lactic acid producer found in the *Rhizopus* genus, while  
*Rhizopus delemar* and *Rhizopus niveus* can produce significant amount of  
extracellular lipase. In addition, *R. oryzae* can also secrete large amount of  
glucoamylase in the solid culture for starch hydrolysis. Therefore, *R. oryzae*  
could be potentially a host for upgrading lactic acid production as well as foreign  
protein production. However, in the current literature, there is very limited  
information available on gene clones as well as gene regulatory elements  
(promoters) for *R. oryzae*. Less than nine gene clone and partial gene  
sequences are reported for *R. oryzae*, which include glucoamylase, ribosomal  
genes, and aspartic proteinase genes.

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The ability to genetically manipulate filamentous fungi largely depends on  
the successfulness to develop the transformation methods and gene expression  
systems. Transformation methods have been developed for filamentous fungi, in  
particular, *Aspergillus nidulans* and *Neurospora crassa*, including others such as  
*Aspergillus niger*, *Aspergillus oryzae*, *Penicillium nalgiovense*. To effectively  
direct the transcription or expression of an interested gene, strong gene

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regulating elements or promoters are required. These promoters can be isolated from the upstream sequences of strongly expressed gene clones.

Phosphoglycerate kinase gene is one of the highly expressed genes found in yeast and filamentous fungi. This gene encodes some of the most abundant mRNA in the yeast cells, accounting for up to 5% of the total cellular protein expression. After the discovery and characterization of *Saccharomyces cerevisiae* gene, other phosphoglycerate kinase genes were also isolated from various fungal species such as *Penicillium chrysogenum* and *Rhizopus niveus* using *S. cerevisiae* phosphoglycerate kinase gene as homologous gene probe. However, only a few of phosphoglycerate kinase gene promoters were isolated and characterized, which were from *S. cerevisiae*, *Trichoderma reesei*, and *R. niveus*, among others.

To genetically manipulate *R. oryzae*, either for the purpose of metabolic pathway modification, conferring necessary traits such as acid tolerance and upgrading of lactic acid production, or producing biocatalyst of interest, high levels of mRNA expression are always desirable. Therefore, there is a need to isolate strong promoter sequences of *R. oryzae* and design/develop expression vectors, harboring the isolated phosphoglycerate kinase gene promoter.

## SUMMARY OF THE INVENTION

The present invention provides the promoter clone discovery of phosphoglycerate kinase gene 1 of a lactic acid-producing filamentous fungal strain, *Rhizopus oryzae*. The isolated promoter can constitutively regulate gene expression under various carbohydrate conditions. In addition, the present invention also provides a design of an integration vector for the transformation of a foreign gene in *Rhizopus oryzae*.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graphic illustrating an inverse PCR method for promoter clone isolation.

5 FIG. 2 is a graphic of a reverse gel image of PCR clones of *R. oryzae* phosphoglycerate kinase 1 promoter.

FIG. 3 is a graphic illustrating the sequence of a phosphoglycerate kinase 1 promoter of *R. oryzae*, SEQ ID: 7.

10 FIG. 4 is a graphic illustrating the homologous comparison of phosphoglycerate kinase 1 promoter sequences between *R. oryzae*, SEQ ID: 9 and *R. niveus*, SEQ ID: 8.

FIG. 5 is a graphic illustrating the expression of phosphoglycerate kinase 1 gene under different conditions.

FIG. 6 is a graphic illustrating a plasmid vector pGA2128 construction.

15 FIG. 7 is a graphic illustrating a plasmid vector pGA2130 construction for *R. oryzae* transformation.

## DESCRIPTION OF THE PREFERRED EMBODIMENT(S)

20 The present invention presents a promoter discovered in a fungal strain, *R. oryzae*, which is a lactic acid producing organism. The discovered promoter is related to the 3-phosphoglycerate kinase gene 1 (pgk1) promoter. The isolated promoter clone, SEQ ID: 7, has a length of 1257 base pairs prior to the pgk1 gene initiation codon. The present invention also shows that the *R. oryzae* pgk1 promoter is a constitutive promoter, which can regulate pgk1 gene expression in various media containing starch, glucose, mannose, galactose, xylose, and arabinose, respectively. In addition, the *R. oryzae* gene 1 has a mature transcription size of about 2.0 kb, based on the Northern blot analysis. This is different from the deduced open reading frame 1251 base pairs of pgk1 gene discovered in *R. niveus*. Further, as compared to the known upstream of *R. niveus* pgk1 gene sequence, SEQ ID: 8, from 1 to 555 base pairs, the *R. oryzae* pgk1 promoter sequence, SEQ ID: 9, from 701 to 1254 base pairs has a

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significant difference of 34 base pairs. In addition, the *R. oryzae* pgk1 promoter has an important TATA box (TAATA) located upstream of ATG at 1187 bp, while the *R. niveus* pgk1 gene does not have this TATA box at this position.

Furthermore, this invention presents a design of a transformation vector for the fungal strain, *R. oryzae*, which utilizes the native pgk1 promoter to regulate the antibiotic (blasticidin) resistance gene of *Aspergillus terreus* in *R. oryzae*. This vector can potentially be used as a chromosomal integration vector for other foreign gene expression in *R. oryzae*. Another objective of the current invention is to use the *R. oryzae* pgk1 promoter to regulate foreign gene expression in other fungal species and plants.

For a clear and concise understanding of the specification and claims, including the scope given to such terms, the following definitions are provided:

PROMOTER: The expression of a gene is directed by a promoter, which is a DNA sequence and locates in the 5' region of a gene. A fungal promoter is a promoter sequence that will direct the transcription of a gene in fungal cells.

CONSTITUTIVE PROMOTER: The rate of gene transcription under such promoter is not regulated by an inducing agent, which can be a chemical compound, or a carbohydrate.

INDUCIBLE PROMOTER: an inducing agent regulates the rate of gene transcription under such promoter.

PLASMID VECTOR: A DNA plasmid vector contains a replicon or an origin of replication able to autonomously replicate the plasmid DNA in the original host organism. A plasmid vector can also serve as both a cloning vector for DNA manipulation in a bacterial host and a shuttle plasmid vector for interested DNA expression in another host cell.

CLONING PLASMID VECTOR: Cloning vectors typically contain one or a small number of restriction endonuclease recognition sites at which interested DNA sequences can be inserted for DNA manipulation purposes. Cloning vectors also contain a marker gene that is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance or ampicillin resistance.

SHUTTLE PLASMID VECTOR: Shuttle plasmid vectors are plasmid vectors that contain two replicons; one of which replicates plasmid vector in a bacterial host cell for DNA manipulation; and the other replicates plasmid vector in another host cell for gene expression. The shuttle plasmid vector typically contains two selectable marker genes; one of which is usually an ampicillin resistance gene or tetracycline resistance used for selection of cells transformed with the vector during DNA manipulation; and the other is usually an anti-fungal antibiotic resistance gene used for the selection of expression host cells transformed with the vector. A shuttle plasmid vector can also be an expression vector and normally contains an expression cassette (promoter//multiple cloning sites//transcription terminator) in which a gene of interest can be inserted.

CHROMOSOMAL INTEGRATION VECTOR: A chromosomal integration vector is a plasmid vector which is able to integrate the whole plasmid DNA or part of the interested DNA into the cell chromosomal DNA. The chromosomal integration is due to recombination of homologous DNA fragment into the cell chromosome by efficient DNA repairing mechanism during fungal transformation. A chromosomal integration vector can also be an expression vector and normally contains an expression cassette (promoter//multiple cloning sites//transcription terminator) in which a gene of interest can be inserted for gene expression or promoter characterization.

**EXAMPLE 1** PCR cloning of phosphoglycerate kinase 1 promoter of *R. oryzae*.

To isolate the phosphoglycerate kinase 1 (pgk1) promoter from *R. oryzae* (ATCC 9363), the *R. oryzae* mycelium was grown overnight in a culture medium containing yeast extract 1%, peptone 2%, and potato starch 2%. Cells were then harvested and genomic DNA was isolated and purified from the culture. Inverse PCR method was used to clone out the promoter region, as shown in Figure 1 where P1 is PCR reverse primer 1; P2 PCR forwarding primer 2; RE restriction enzyme site which can be cleaved both upstream of the pgk1 promoter and inside of the pgk1 gene; RO *Rhizopus oryzae*. PCR primers for the inverse PCR were designed based on the open reading frame of the pgk1 gene sequence of *Rhizopus niveus* under the assumption that pgk1 gene of *R. oryzae* and *R.*

*niveus* is homologous. A 5' end over-hung sequence (*italics*) was designed to adapt restriction enzyme sites (underlined) such as Xba I and Sph I. The inverse PCR primers are listed as following:

5 Reverse primer PGK1-C-97; SEQ ID 1:

GC TCT AGA AGG TTG AGG TCG CGA ATA GAG AGC TTG

Reverse primer PGK2-C-98; SEQ ID 2:

GC TCT AGA ACG GTA GGA AGA GCT TGA ACG ATA CGA

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Forwarding primer PGK3-N-99; SEQ ID 3:

GAT GCA TGC TCT CAA ACC AGT GGC TGC TGA GGT TGA

Forwarding primer PGK4-N-100; SEQ ID 4:

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GAT GCA TGC GCC TTT GGT ACT GCT CAC CGT GCT CAC

Forwarding primer PGK5-N-101; SEQ ID 5:

GAT GCA TGC TGT TAA AGT AAG GTT CTC TTA TAA

20 Forwarding primer PGK6-N-102; SEQ ID 6:

GAT GCA TGC TAT TTG AAT TCG ATG CCT TCT CTA

The genomic DNA is first digested with different restriction enzymes, including Bgl II, EcoR I, Hinc II, Kpn I, Nco I, Sph I, and Xmn I, which locate  
25 within the 5' region of the *pgk1* gene of *R. niveus*. After digestion, the DNA samples are then purified and self-ligated using T4 DNA ligase. Table 1 shows the reverse PCR reaction matrix, which pairs various sets of reverse primer and forwarding primer together.

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Table 1. Inverse PCR primer pair sets correspondent to each restriction enzyme digested DNA sample for the isolation of 3-phosphoglycerate kinase 2 promoter.

PCR reaction No.	Restriction enzyme used for DNA digestion	Inverse PCR primer pairing
1	Bgl II	PGK1-C-97; PGK1-C-101
2	Bgl II	PGK1-C-98; PGK1-C-101
3	EcoR I	PGK1-C-97; PGK1-C-99
4	EcoR I	PGK1-C-98; PGK1-C-99
5	Hinc II	PGK1-C-97; PGK1-C-100
6	Hinc II	PGK1-C-98; PGK1-C-100
7	Kpn I	PGK1-C-97; PGK1-C-102
8	Kpn I	PGK1-C-98; PGK1-C-102
9	Nco I	PGK1-C-97; PGK1-C-100
10	Nco I	PGK1-C-98; PGK1-C-100
11	Sph I	PGK1-C-97; PGK1-C-99
12	Sph I	PGK1-C-98; PGK1-C-99
13	Xmn I	PGK1-C-97; PGK1-C-99
14	Xmn I	PGK1-C-98; PGK1-C-99

The inverse PCR reactions were conducted based on the primer pairing outlined in Table 1. After PCR reaction, the PCR products were separated in an agarose gel by electrophoresis. The inverse PCR results are shown in a reverse gel image in Figure 2, where lane number is correspondent to each inverse PCR reaction in Table 1 and lane H $\lambda$  is the DNA size marker. The isolated pgk1 promoter clones are indicated as dark bands in the gel picture. Lanes 3, 4, 5, 6, 9, and 10 show strong bands, which correspondent to the ligated DNA samples previously cleaved by EcoR I, Hinc II, and Nco I, respectively. The sizes of the PCR clones range from about 1.0 kb to 3.0 kb.

**EXAMPLE 2** Nucleotide sequence of pgk1 promoter sequence.

PCR product No. 3 contains most of the pgk1 gene upstream sequence since the restriction enzyme EcoR I used to cleave the genomic DNA locates closer to the initial codon ATG than other restriction enzymes, Hinc II and Nco I, which locate further down stream of EcoR 1 site. The pgk1 promoter clone No. 3 was cloned into a vector pGEM-T (Promega, Madison, WI) to form pGA2086. Individual colonies were picked to confirm DNA insertion. Two of the individual clones, pGA2086a and pGA2086b, were completely sequenced from both ends. By sequence blasting, the overlapping sequence of these two clones indicated that they are identical and belong to one gene sequence. The complete nucleotide sequence of pgk1 gene promoter is shown in Figure 3; SEQ ID 7. The cloned pgk1 promoter sequence has a length of 1257 bp. The putative TATA box and CAT box are bold and underlined. There are six CAT boxes and two TATA boxes within 200 base pairs upstream of the initial codon.

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**EXAMPLE 3** Homologous comparison of pgk1 promoter sequence between *R. oryzae* and *R. niveus*.

To compare the homology of pgk1 promoter between *R. oryzae* and *R. niveus*, promoter sequences from both origins were blasted against each other. The comparison results of pgk1 promoter are shown in Figure 4, where ROPK15 is *R. oryzae* pgk1 promoter sequence, SEQ ID: 9; RNPGK1 is *R. niveus* pgk1 promoter sequence, SEQ ID: 8. The bold letters indicate the difference of both sequences and "-" indicates missing nucleotides of both sequences. ATG is the putative initial codon of pgk1 gene. Results indicate that there are 34 nucleotides of *R. oryzae* pgk1 promoter different from the pgk1 promoter of *R. niveus*. Within the 200 base pairs upstream of ATG, *R. oryzae* pgk1 promoter has two TATA boxes while the one of *R. niveus* has only one TATA box. *R. niveus* does not have the TATA box at 1187 base pair as shown in Figure 4. These results conclude that the pgk1 promoter sequences of *R. oryzae* are not identical to the one isolated from *R. niveus* even though they are highly homologous.

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**EXAMPLE 4** Expression patterns of *pgk1* gene in *R. oryzae*.

To test the regulatory patterns of the *pgk* promoter, *R. oryzae* was grown in different culture media containing glucose, starch, xylose, mannose, galactose, and arabinose, respectively. Total RNA was isolated from the mycelia biomass using an RNeasy Maxi kit (QIAGEN Inc., Valencia, CA). Total RNA samples (50 µg per lane) were separated in 1.3% formaldehyde-agarose gel and capillary-blotted onto Zeta-Probe membrane (Bio-Rad Laboratories, Hercules, CA), which was then baked at 80°C in a vacuum oven for two hours. Northern blot were hybridized with a  $\alpha^{32}\text{P}$ -dCTP labeled *pgk1* gene probe. The Northern blot analysis results are shown in Figure 5, where lane 1, 2, 3, 4, 5, and 6 are total RNA samples prepared from *R. oryzae* cultures grown in the medium with glucose, potato starch, xylose, mannose, galactose, and arabinose, respectively. The Northern blot indicates that *R. oryzae* phosphoglycerate kinase 1 gene has a mature RNA size of about 2 kb, different from the open reading frame (1.25 kb) of *R. niveus* *pgk1* gene reported by indicating that *pgk1* gene of *R. oryzae* is different from the one of *R. niveus*. *R. oryzae* *pgk1* gene expresses in all the conditions tested, indicating that *pgk1* promoter is a constitutive promoter. The strong expression was obtained in the medium containing galactose, glucose, and xylose, followed by arabinose, mannose and potato starch.

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**EXAMPLE 5** Transformation vector design for *R. oryzae*.

To develop transformation vector system for foreign gene expression in *R. oryzae*, the following vector is designed. A blasticidin resistance gene (Invitrogen, Carlsbad, CA) was first cloned out by PCR, and cloned into pGEM-T vector (Promega, Madison, WI) to adapt EcoR I at the 5' end of the gene and Not I site at the 3' end, forming a plasmid vector pGA2125. The blasticidin gene was subsequently cloned into a vector pRG1 as shown in Figure 6, forming plasmid pGA2128. Plasmid vector pRG2130 for gene expression and integration in *R. oryzae* was then constructed and is shown in Figure 7, where AmR: ampicillin resistance gene; bsd: antibiotic blasticidin resistance gene; ori: Col E1 origin; T: fungal transcription terminator  $T_{\text{AOX1}}$ ; pk P: *R. oryzae* *pgk 1* promoter; and URA3: *Pichia pastoris* orotidine-5'-phosphate decarboxylase gene. The selectable

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marker gene provides *R. oryzae* with resistance to antibiotics, blasticidin. Other antibiotic resistance genes such as sulfanilamide and Gentamycin resistance genes can replace the blasticidin selectable marker gene. The selectable marker gene is placed under the control of *R. oryzae* pgk1 promoter and a fungal transcription terminator, T<sub>AOX1</sub>, terminates the transcription. In addition, a heterogenous or homogenous URA3 gene or other native gene sequences can be used as integration elements for chromosomal gene insertion. Also, pgk1 promoter sequence can potentially serve as chromosomal integration elements. Chromosomal integration vectors incorporate the desired gene into cell chromosome based on the underlying principle that linearized plasmid DNA fragments are efficiently repaired during fungal transformation by recombination with a homologous DNA restriction fragments.

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## CLOSURE

While preferred embodiments of the present invention have been shown and described, it will be apparent to those skilled in the art that many changes and modifications may be made without departing from the invention in its broader aspects. The appended claims are therefore intended to cover all such changes and modifications as fall within the true spirit and scope of the invention.

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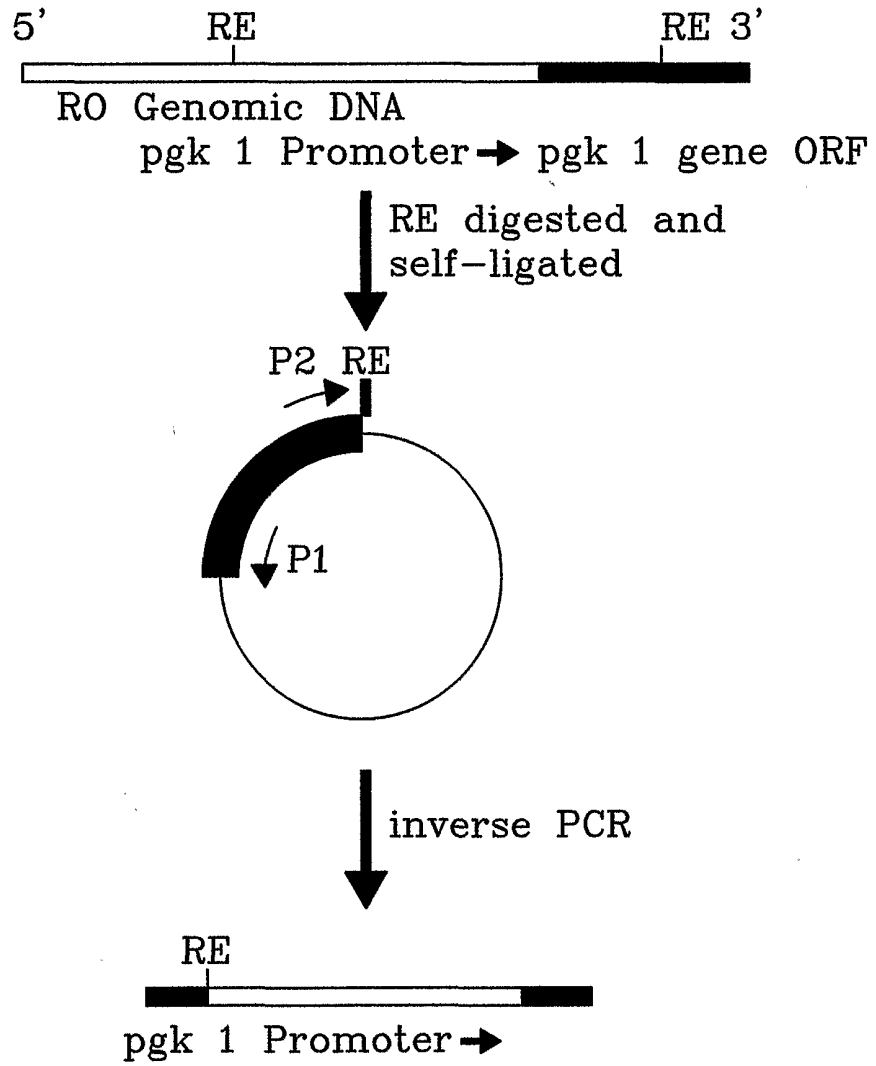
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## CLAIMS

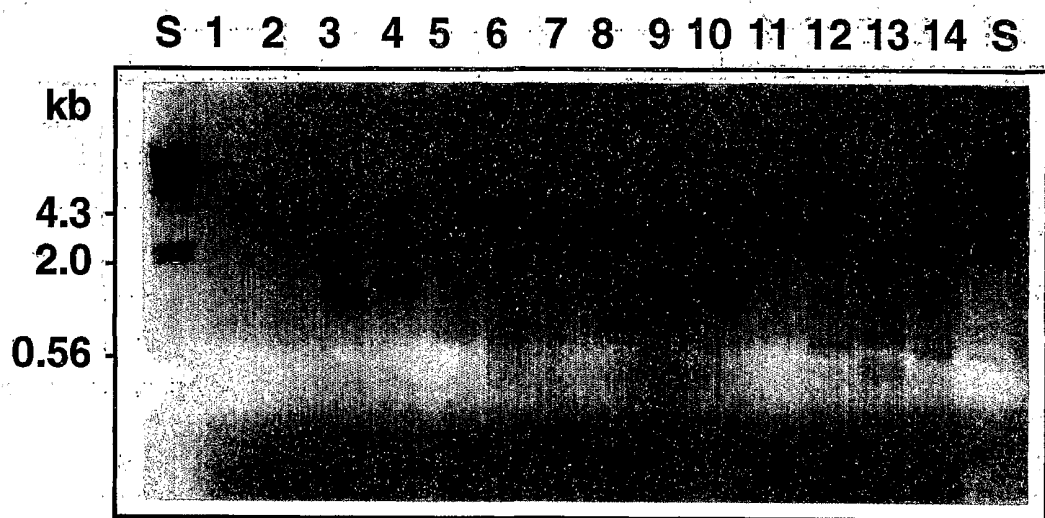
We claim:

1. An isolated fungal promoter, which is native to *Rhizopus oryzae* and  
5 located upstream of and in control of a phosphoglycerate kinase gene 1.
2. The isolated fungal promoter of claim 1, wherein said promoter has a  
sequence of 1254 base pairs prior to the initiation codon of pgk1 gene.  
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3. A vector comprising the fungal promoter of claim 1.
- 15 4. The vector of claim 3, whereby the vector is a plasmid vector.
5. The vector of claim 4, whereby the plasmid vector is a chromosomal  
integration vector.  
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6. A process of expressing a gene of interest in fungal species, comprising  
the steps of:
  - a. fusing a gene of interest to an isolated fungal promoter, which is  
native to *Rhizopus oryzae* and located upstream of and in control of a  
25 phosphoglycerate kinase gene 1;
  - b. integrating the said fungal promoter and a gene of interest within a  
fungal genomic DNA such that the said promoter regulates the expression of  
the gene of interest in fungal cells/mycelia.
- 30 7. A process of expressing a gene of interest in fungal species, comprising  
the steps of:

- a. Fusing a gene of interest to an isolated fungal promoter, which is native to *Rhizopus oryzae* and located upstream of an in control of a phosphoglycerate kinase gene 1;
- b. constructing a plasmid vector comprising said fused gene;
- 5 c. replicating said plasmid vector within a fungal cell/mycelia such that the said fungal promoter regulates the expression of the gene of interest in the fungal cells/mycelia.



*Fig. 1*



**Fig. 2**



1 tctcaaaccagtggtgctgaggttgaaaagcttttgggaaaacctgtcgaattcaagct 60  
61 tctgaggatgctatcttagctgcaaagcatggttgcaaggtattgtcatctcaacat 120  
121 ggtggtagacaattggatttcgcaccttctgctattgaaatcttgcctgaagtcacagct 180  
181 gctcttaagcgaggaagaattaatgaagaattgaggtttatattgatggaggtattcga 240  
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1201 agcaataacttttcttctcttctcttaattcaataccaattttcaacagaATG 1257

*Fig. 3*

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RNP GK1:1          tacattatat 10
ROPK15:701        atatttgtac 710

RNP GK1:11        tatgtgagcataacaatctatttatattgctttattataacttagataacttactgatac 70
ROPK15:711        tatatgagcacaacaatctatttataactgccttattacagtttagacaacttactgatac 770

RNP GK1:71        gcattgattattctttgtacgcagacactaaccaaacaggttagctttatacctacctgt 130
ROPK15:771        tcattgattattctttgtacgcagacactaaccaaacacattagttttataccaacctgt 830

RNP GK1:131       gaat t t t a t a a t t c a g c t t g c t t a a g c t t t c c a a g t g g c a t g g t t t t t t c t c t g a t g t c g 190
ROPK15:831       gaat t t t a t a a t t c a g c t t g c t t a a g c t t t c c a a g t g g c a t g g t t t t t t c t c t g a t g t c g 890

RNP GK1:191       c g a t a t c a a a a t t c a a g a c c g t a t c a c a t t t a t c a a c a a a a a a a a g g t g a c t c c a a g 250
ROPK15:891       c g a t a t c a a a a t t c a a a c c g t a t c a c a t c t a t c a a a c - a a a a a a a a a g g t g a t t c c a a g 949

RNP GK1:251       a t g t t a t c a g a a a t a t c a a g c c a a g t t t a c - t t t a t a t t a t t c g t t t a g g a a g c t t c a t t 309
ROPK15:950       a t g t t a t c a g a a a t a t c a a g c t a g g c t t a c t t t t a t g t t a t t c g t t t a g g a a g c t t c a t t 1009

RNP GK1:310       t t t t a g a a g a g a a a a a a a a g a a g t t g a a a t a c a a g g a a c a a g t t a a a t g t t a t a c 369
ROPK15:1010      t t t t a g a a g a g a a a a a a a a a g a g t t g a a a t a c a a g g a a c a a g t t a a a t g t t a t a c 1066

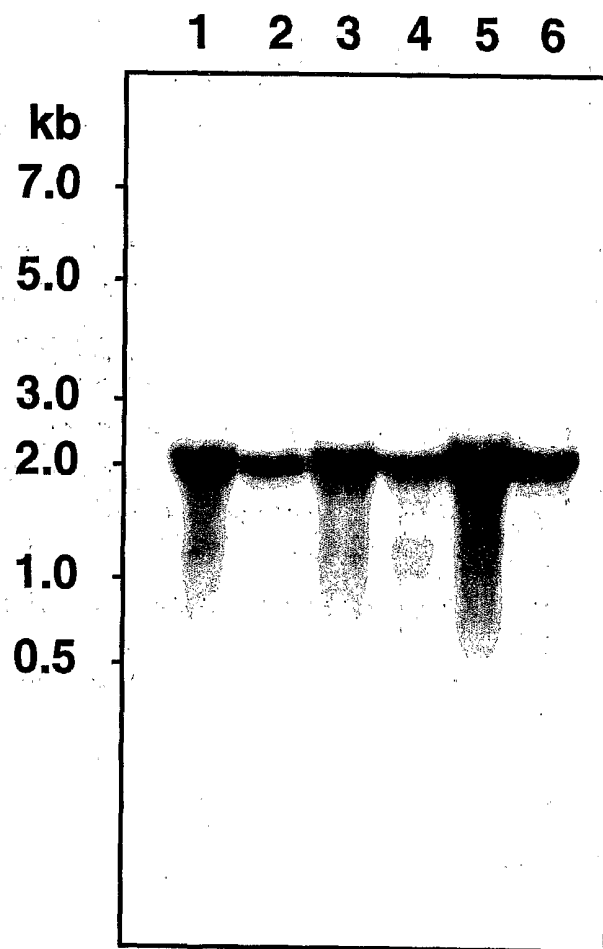
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ROPK15:1067      a a t a a g c a t g t g c t t t c a t a a c a g a a a g g c t c c t t t a g t t t a a t g a a a t c c c g a t t t c c a 1126

RNP GK1:430       t c g a a c a a t t a c c c a a t a a t a t c a t t t g g g a a a g t g a a g t c a t g t a t g a t t a t t t t t t c 489
ROPK15:1127      t c g a a c a a t t a c c c a a t a a t a t t a t t t g g g a a a g t g a a g t c a t g t a t g a t t a t t t t t t c 1186

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Fig. 4



**Fig. 5**

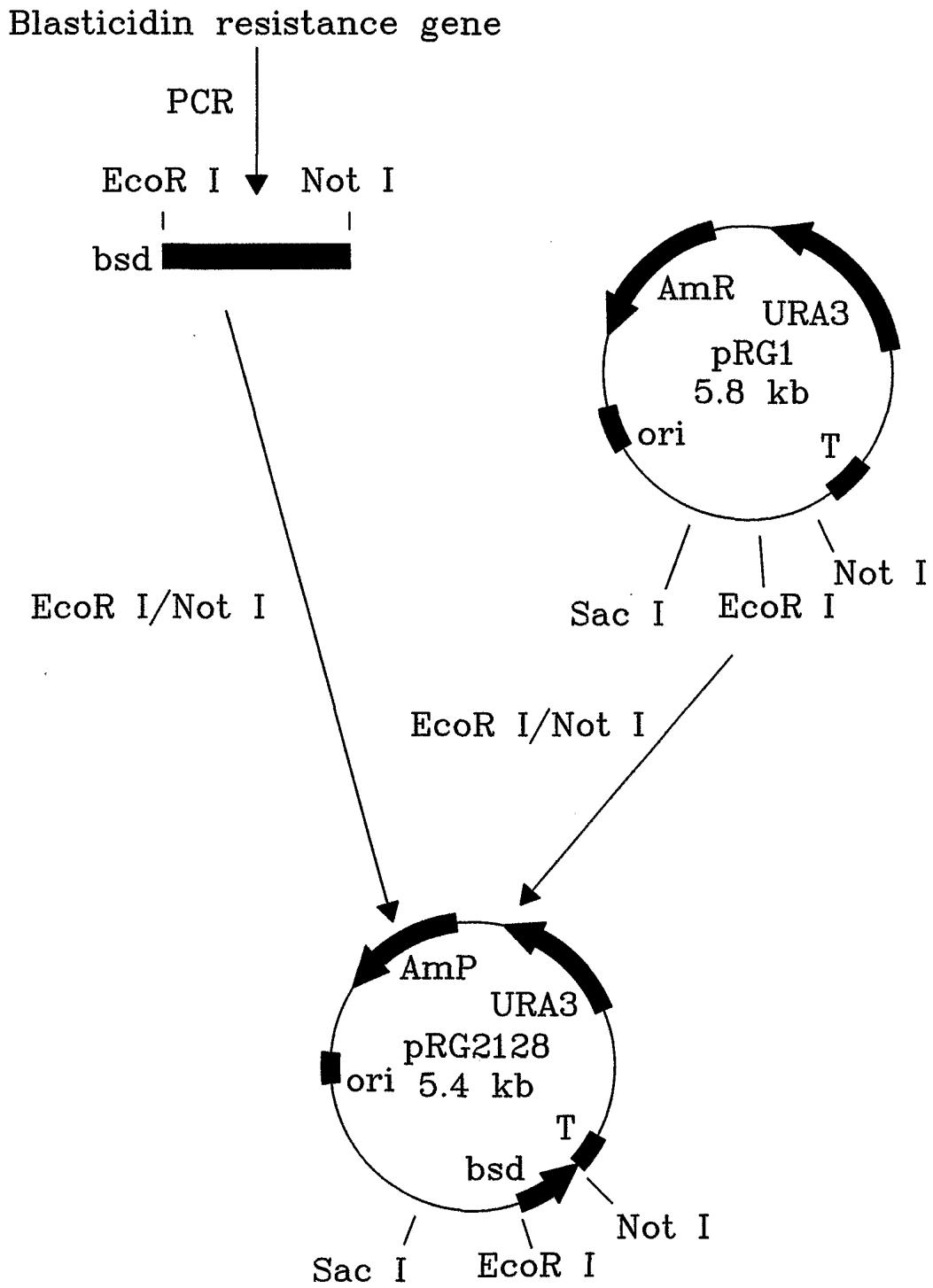
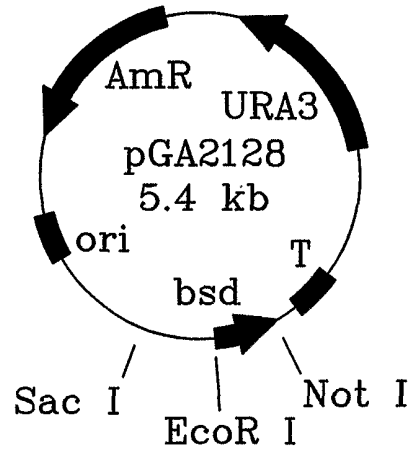


Fig. 6

R. oryzae pgk1 promoter  
in pGA2086a

PCR

Sac I ↓ EcoR I



Sac I/EcoR I

Sac I/EcoR I

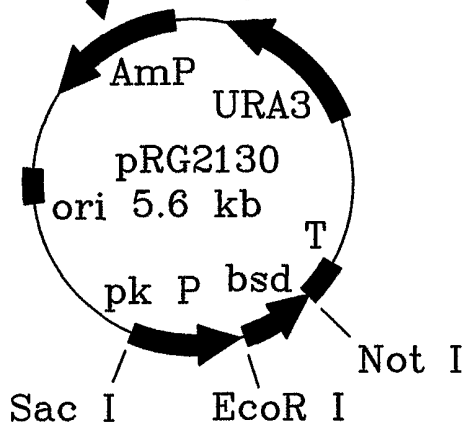


Fig. 7

## SEQUENCE LISTING

<110> Battelle Memorial Institute

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Method of Expressing a Gene of Interest in Fungal  
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