KURENAI 紅
Kyoto University Research Information Repository

KYOTO UNIVERSITY

Title	Characterization of galactose-dependent promoters from an oleaginous fungus Mortierella alpina 1S-4.
Author(s)	Okuda, Tomoyo; Ando, Akinori; Sakuradani, Eiji; Kikukawa, Hiroshi; Kamada, Nozomu; Ochiai, Misa; Shima, Jun; Ogawa, Jun
Citation	Current genetics (2014), 60(3): 175-182
Issue Date	2014-02-19
URL	http://hdl.handle.net/2433/198767
Right	The final publication is available at Springer via http://dx.doi.org/10.1007/s00294-014-0422-5.
Type	Journal Article
Textversion	author

Kyoto University

Characterization of galactose-dependent promoters from an oleaginous fungus *Mortierella alpina* 1S-4

Tomoyo Okuda · Akinori Ando · Eiji Sakuradani · Hiroshi Kikukawa · Nozomu Kamada · Misa Ochiai · Jun Shima · Jun Ogawa*

Tomoyo Okuda, Akinori Ando, Eiji Sakuradani, Hiroshi Kikukawa, and Jun Ogawa
Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto 606-8052, Japan

Akinori Ando
Research Unit for Physiological Chemistry, Kyoto University, Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto 606-8052, Japan

Nozomu Kamada and Misa Ochiai
Research Institute, Suntory global Innovation Center Ltd., 1-1-1 Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka 618-8503, Japan

Jun Shima
Research Division of Microbial Sciences Kyoto University, Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto 606-8052, Japan

Corresponding author: Jun Ogawa
Tel: +81 75 753 6113
Fax: +81 75 753 6128
E-mail: ogawa@kais.kyoto-u.ac.jp

Abstract

An inducible promoter is a useful tool for the controlled expression of a given gene. In this report, we describe galactose-dependent promoters for potential use in an oleaginous fungus *Mortierella alpina*. We cloned the putative promoter regions of two genes encoding galactose metabolic enzymes, GAL1 and GAL10, from the genome of *M. alpina* 1S-4. The β-glucuronidase (*GUS*) reporter gene assay in *M. alpina* 1S-4 revealed that regulation of these promoters was dependent on the presence of galactose in the medium both with and without other sugars. With the *GAL10* promoter, an approximately 50-fold increase of GUS activity was demonstrated by addition of galactose into the culture media at any cultivation phase. The 5′ deletion analysis of the *GAL10* promoter revealed that a promoter region of over 2,000 bp length was required for its high-level activity and sufficient inducible response. Significantly, this is the first report of inducible promoters of zygomycetes. The *GAL10* promoter will be a valuable tool for gene manipulation in *M. alpina* 1S-4.

Introduction

An inducible expression system is an significantly important tool for the control of gene expressions. It is necessary for the expression analysis of given genes, especially lethal and essential genes. Many investigations of inducible expression system have been carried out in various microorganisms (Cereghino and Cregg 2000, Punt, et al. 2002, Terpe 2006). Some of the most widely used regulatory systems are based on promoters that can be activated or repressed by the presence/absence of the inducer such as a carbon source in the medium. In *Escherichia coli*, for example, the lactose-inducible promoter for the *lac* operon traditionally has been used to control the cloned genes of interest (Amann, et al. 1983). In *Saccharomyces cerevisiae*, the galactose-inducible expression system using the galactokinase (*GAL1*) and uridine diphosphate glucose 4-epimerase (*GAL10*) promoters has been commonly used (Johnston and Davis 1984). In the methylotrophic yeast *Pichia pastoris*, various medically important proteins have been produced by using strong methanol-inducible promoter (Cereghino and Cregg 2000, Hansson, et al. 1993, Sumi, et al. 1999). Also in fungus, mainly in *Aspergillus* species, amylose-, xylose- and pyruvate-inducible systems have been established and applied (Archer, et al. 1994, Ichishima, et al. 1999, MacKenzie, et al. 1993, Punt, et al. 2002). These inducible expression systems have

contributed to the functional analysis of genes of interest as well as for the efficient production of the heterologous proteins in these microorganisms.

An oleaginous fungus *Mortierella alpina* 1S-4, belonging to zygomycetes, is a microbe that highly produces polyunsaturated fatty acids (PUFAs) (Shimizu, et al. 1997). To modify the PUFA biosynthetic pathway and to improve PUFA productivity, basic molecular breeding tools such as gene delivery systems, host-vector systems and transformation systems using auxotrophy or antibiotic resistance were established in *M. alpina* 1S-4 (Ando, et al. 2009a, Takeno, et al. 2004a, Takeno, et al. 2005). Due to the demand for functional lipids with beneficial effects on human health (Gill and Valivety 1997), the production of various PUFAs has been achieved by molecular modification of *M. alpina* (Jareonkitmongkol, et al. 1992, Jareonkitmongkol, et al. 1993, Kawashima, et al. 1997, Sakuradani, et al. 2013). However, the molecular knowledge of *M. alpina* is still incomplete because of the lack of inducible systems in this strain. At present, a few constitutive expression promoters have been identified and applied to the gene expression system of *M. alpina*. The lack of an inducible expression system in *M. alpina* limits of detailed study of genes of interest, especially essential or lethal genes. To increase knowledge of *M. alpina*, it is essential to establish an inducible expression system.

Here, we report the cloning and initial characterization of endogenous galactose inducible promoters for use in *M. alpina* 1S-4.

Materials and Methods

Strains, media, and growth conditions

A uracil auxotroph (*ura5⁻* strain), previously isolated from *M. alpina* 1S-4 deposited in the Graduate School of Agriculture of Kyoto University (Takeno, et al. 2004b), was used as a recipient host strain for transformation. Czapek-Dox agar medium, supplemented with 0.05 mg/ml uracil, was used for sporulation of the *ura5⁻* strain, as described previously (Takeno, et al. 2004b). SC medium (Takeno, et al. 2004b), was used as a uracil-free synthetic medium for cultivation of the transformants derived from *M. alpina* 1S-4 *ura5⁻* strain at 28°C. GY medium (2% [wt/vol] glucose and 1% yeast extract) was used as a rich medium. For galactose induction in the submerged cultivation, 500 mg/ml sterile galactose solution was added to the medium at 2% final concentration.

E. coli strain DH5α was used for DNA manipulation and grown on LB agar plates containing 50 μg/ml kanamycin.

Agrobacterium tumefaciens C58C1 was used for the transformation of *M. alpina* 1S-4 *ura5⁻* strain. LB-Mg agar medium, minimal medium (MM), and induction medium (IM) were used for transformation, cultivation and infection of *A. tumefaciens*, respectively.

6

The compositions of LB-Mg agar medium, MM and IM were described previously (Takeno, et al. 2004b).

Liquid cultivations were performed at 28°C with shaking at 300 rpm. Solid media contained 2% agar.

Genomic DNA preparation

M. alpina 1S-4 was cultivated in 10 ml of GY medium at 28°C for 4 days with shaking at 300 rpm. Fungal mycelia were harvested by suction filtration and washed twice with sterile water. Preparation of genomic DNA was performed by employing the method described previously (Sakuradani, et al. 1999).

Construction of GUS *reporter gene-carrying vectors for promoter analysis*

The reporter gene vectors were constructed on the backbone of pBIG3ura5s (Ando, et al. 2009b). The histone promoter (the histone H4.1 promoter short fragment (Ando, et al. 2009b)), succinate dehydrogenase subunit B (*SdhB*) terminator (Ando, et al. 2009a) and *ura5* marker gene (Takeno, et al. 2004a) were amplified from genomic DNA of *M. alpina* 1S-4. The *ura5* expression cassette, controlled by the histone promoter and *SdhB* terminator, was generated by fusion PCR with additional *Eco*RI and *Xba*I restriction

enzyme sites at the 5′ and 3′ end of this cassette, respectively. The *ura5* expression cassette was digested with *Eco*RI and *Xba*I and ligated to pBIG3ura5s (Ando, et al. 2009b), which had been digested with the same restriction enzymes, and designated as pBIG35Zh.

The *β*-Glucuronidase (*GUS*) gene was synthesized with optimized codon usage to reflect the codon bias of *M. alpina* 1S-4 obtained from the Kazusa database (http://www.kazusa.or.jp/codon/), with additional *Spe*I and *Bam*HI restriction enzyme sites at the 5′ and 3′ flanking open reading frame (ORF), respectively. The *GUS* expression cassette, controlled by the histone promoter and *SdhB* terminator, was generated by fusion PCR with additional *Xba*I and *Nhe*I restriction sites at the 5′ and 3′ end of the cassette, respectively. This *GUS* expression cassette was digested with *Xba*I and *Nhe*I and ligated to pBIG35Zh, which had been digested with the same restriction enzymes and designated pBIG35ZhGUSm. On this vector, the histone promoter region located upstream of the *GUS* gene could be removed by digestion with *Xba*I and *Spe*I and replaced by another promoter fragment for a promoter assay.

The *GAL1* and *GAL10* promoter regions were amplified from the genome of *M. alpina* 1S-4 by PCR with specific primers (Table 1) designed based on the genomic database of this strain. For deletion constructs of the *GAL10* promoter, GAL10pR was used as the

anti-sense primer, and GAL10p2000F, GAL10p1600F, GAL10p1200F, GAL10p800F

and GAL10p400F were used as the sense primers (Table 1). All cloned fragments were

treated with *Xba*I and/or *Spe*I and inserted in front of the GUS ORF from

pBIG35ZhGUSm digested with *Xba*I and *Spe*I.

Transformation of the M. alpina *1S-4 ura5⁻ strain*

A spore suspension of the *M. alpina* 1S-4 *ura5⁻* strain was freshly prepared by

harvesting from cultures growing on Czapek-Dox agar medium supplemented with 0.05

mg/ml uracil and then filtering the suspension through Miracloth (Calbiochem,

Darmstadt, Germany) (Takeno, et al. 2004b).

Transformation of the *M. alpina* 1S-4 *ura5⁻* strain was performed with the

Agrobacterium tumefaciens-mediated transformation (ATMT), with slight modification

of the previously described method (Ando, et al. 2009b). Initially, *A. tumefaciens*

C58C1 was transformed with each vector via electroporation, as described previously

(Shen and Forde 1989), and its transformants were isolated on LB-Mg agar plates

supplemented with kanamycin (20 μg/ml), ampicillin (50 μg/ml) and rifampicin (50

μg/ml). *A. tumefaciens* transformants were cultivated in 100 ml of MM supplemented

with kanamycin (20 μg/ml) and ampicillin (50 μg/ml) at 28°C for 48 h with shaking at

120 rpm. Bacterial cells were harvested by centrifugation at 8,000 × g, washed once with fresh IM, and then diluted to an optical density of 660 nm (OD_{660}) of 0.1–0.2 in 10 ml of fresh IM. After pre-incubation for 12–16 h at 28°C with shaking (300 rpm) to an OD_{660} of 1.5–2.0, 100 µl of the bacterial cell suspension was mixed with an equal volume of *M. alpina* 1S-4 *ura5*⁻ spore suspension (10^8 spores/ml), and then spread on membranes (Whatman 50 Hardened Circles 70 mm; Whatman International Ltd., UK) placed on co-cultivation media (IM with 1.5% agar) and incubated at 23°C for 5 days. After co-cultivation, the membranes were transferred to uracil-free SC agar plates that contained 0.03% Nile blue A (Sigma-Aldrich Japan) to discriminate between fungal colonies and the white color of the membrane. After 2 days of incubation at 28°C, hyphae from visible fungal colonies were transferred to fresh uracil-free SC agar plates, and this transfer, was repeated three times to obtain transformation candidates. Integration of the vector into the host chromosome was verified by PCR, as described previously (Takeno, et al. 2004b).

Preparation of cell-free extracts for GUS assay

Cell-free extracts of *M. alpina* were prepared by a slight modification of the method described previously (Takeno, et al. 2004a). For solid cultivation, all transformants of *M.*

alpina 1S-4 were cultivated on the agar medium for 3 days at 28°C and harvested by scratching. For liquid cultivation, all transformants were cultivated in 10 ml of liquid medium for 2–14 days at 28°C with shaking at 300 rpm, harvested by suction filtration and washed twice with sterile water. Harvested fungal mycelia were suspended in 2 volumes of 100 mM Tris-HCl containing 5 mM 2-mercaptoethanol (pH7.5) and then twice disrupted by using a bead shocker (Wakenyaku Co., Ltd., Kyoto, Japan) at 5,000 rpm for 30 s with glass beads (φ 1.0 mm; Waken B Tech Co., Ltd., Kyoto, Japan). The extract was centrifuged at 15,000 × g for 10 min to remove cell debris and intact cells. The supernatant was used for the GUS assay as cell-free extract. All steps were performed at 4 °C.

GUS assay and protein measurement

GUS assays were performed as described previously (Jefferson, et al. 1986). The enzyme activity was calculated in terms of nanomoles of *p*-nitrophenol production per milligram of protein per minutes at 37°C. The protein concentration was measured according to the Bradford assay, using bovine serum albumin as standard (Bradford 1976).

Nucleotide sequence accession number

The sequences of *GAL1* and *GAL10* promoters were deposited in DNA Data Bank of

Japan (DDBJ) under the accession numbers AB871458 and AB871459, respectively.

Results

Cloning and basic evaluation of two GAL *promoters in* M. alpina *1S-4*

The putative promoter region of the *GAL1* and *GAL10* genes of *M. alpina* 1S-4 were cloned as candidates for galactose-dependent promoters based on the *M. alpina* genome database. The lengths of cloned *GAL1* and *GAL10* promoter regions were 962 bp and 2331 bp, respectively.

To ascertain if these promoters were regulated by galactose in this strain, plasmids carrying the predicted *GAL1* and *GAL10* promoters fused to the *GUS* reporter gene were constructed and transformed into *M. alpina* 1S-4 by the ATMT method. All transformants had a single copy of T-DNA at a random location in the chromosomal DNA (data not shown). At least 30 independent transformants for each construct were randomly selected, cultivated on SC medium containing 2% galactose substituted for glucose, and evaluated for GUS activity. All transformants exhibited detectable levels of GUS activity (data not shown), and three individual transformants that showed moderate levels of GUS activity were used in subsequent studies.

The transformants carrying *GAL1* or *GAL10* promoter-*GUS* genes were cultivated on SC agar medium containing 2% of sugars substituted for glucose (Table 2). As shown in

Table 2, the expression of *GUS* regulated by the *GAL* promoters was clearly dependent on the presence of galactose in the medium. The GUS activity of fungi with *GAL1* or *GAL10* promoters grown on galactose medium was approximately 7-fold or 100-fold higher than those grown on glucose medium, respectively. With the *GAL1* promoter, GUS expression was induced by galactose, lactose and raffinose; furthermore, the not-negligible level of GUS activity was detected even when grown on the medium without a carbon source. On the other hand, GUS expression with the *GAL10* promoter was fairly repressed when fungi were grown on media lacking galactose with/without other kinds of sugars.

Because *GUS* expression with the *GAL10* promoter were more sensitively induced/repressed by the presence/absence of galactose, we focused on the *GAL10* promoter for further investigation.

Induction-response of the GAL10 *promoter by galactose addition*

Time course measurements of *GAL10* promoter activity after addition of galactose to the medium were carried out. The transformants were cultivated for 4 days in synthetic SC liquid medium containing 2% raffinose substituted for glucose as a sole carbon source, and then galactose (2% final concentration) was added to the medium. The GUS

activity was monitored over a 48-h time course (Fig. 1). An increase in GUS expression

was detected at 10 h after the addition of galactose, and then the GUS expression level

reached a peak at 36 h.

As shown in Fig. 2, GUS expression was induced by the addition of galactose,

regardless of cultivation phase of mycelia. In all cases, the induction of GUS expression

was maintained for 2–3 days, and then GUS activity declined.

Induction-response of the GAL10 *promoter in complex medium*

We also investigated the induction of expression by the *GAL10* promoter in the

nutrient rich medium. The transformants were cultivated in GY liquid medium (2%

glucose and 1% yeast extract), and then galactose was added at day 4, 7 or 10 during the

cultivation (Fig. 3). As shown in Fig. 3, GUS activity was induced by the addition of

galactose in the same manner as that observed with synthetic medium. In all cases, the

induction of GUS expression was maintained for approximately 3 days, and then GUS

activity declined.

Deletion analysis of the GAL10 *promoter*

In order to investigate the length of promoter regions required to induce high

expression, a series of 5′ *GAL10* promoter deletion constructs was generated (Fig. 4, left) and introduced into *M. alpina* 1S-4. For each deletion constructs, 30 independent transformants were randomly selected, cultivated on SC medium containing galactose substituted for glucose, and evaluated for GUS activity. For comparison, 10 individual transformants that showed moderate levels of GUS activity were selected, cultivated in presence/absence galactose in SC medium containing raffinose substituted for glucose, and then evaluated for GUS activity (Fig. 4, center). As shown in Fig. 4, a relatively long promoter region (over 2,000 bp) was required to induce sufficient GUS expression. GUS activity dramatically diminished with the deletion of 5′ regions. Responsiveness to galactose was also declined with the deletion, but a certain level of fold induction (13.5-fold) was kept even in a shorter region (1,601 bp) (Fig. 4, right).

Discussion

In this study, we investigated the promoter regions of the *GAL1* and *GAL10* genes as inducible promoter candidates for an oleaginous fungus *M. alpina*. The enzymes coded by these genes are involved in the galactose-metabolic pathway; GAL1 catalyzes phosphorylation of galactose, and GAL10 catalyzes epimerization from uridine diphosphate galactose to uridine diphosphate glucose. The promoter regions of genes homologs to of *GAL1* and *GAL10* have been reported as galactose-inducible promoters in various microorganisms including *Saccharomyces cerevisiae* (Johnston and Davis 1984). Promoters of other genes involved in the galactose-metabolic pathway, such as *GAL4* and *GAL7*, also have been used as inducible promoters in such microorganisms (Chien, et al. 1991, Giniger, et al. 1985, Griggs and Johnston 1991, Johnston 1987, Wickes and Edman 1995). Although *GAL4* and *GAL7* homologs were not found in the *M. alpina* genome database, discovery of these genes could result in isolation of other galactose-inducible promoters.

In general, useful inducible promoters exhibit the following features: (i) easily controlled by the presence or absence of components in the medium and (ii) fully repressed in the absence of inducer in the medium. For the convenience of induction in submerged cultivation, we searched for another useful feature of an inducible promoter:

(iii) induced by addition of the inducer into the medium, rather than by replacement of medium. The GUS reporter assay revealed that *GAL1* and *GAL10* promoters were both regulated by the presence/absence of galactose in the medium (Table 2). In particular, GUS activity regulated by the *GAL10* promoter was extremely low in the medium without galactose. On the other hand, GUS activity was detectable even when other sugars were present in the medium containing galactose (Table 2). This result suggests that the *GAL10* promoter activity can be fully repressed during cultivation in the medium containing sugars other than galactose, and then easily induced by the addition of galactose into the medium. Therefore, we focused on *GAL10* promoter and carried out further investigation.

To investigate the function of the *GAL10* promoter in submerged cultivation, we used raffinose as a sole carbon source in pre-culture medium, because raffinose did not affect the induction of the *GAL10* promoter in medium with/without galactose (Table 2). When galactose was added to the medium, in which transformants with the *GAL10* promoter fused with the *GUS* gene were pre-cultivated, GUS activity was elevated 10 h after the addition of galactose (Fig. 1), was maintained for 2–3 days, and then declined (Fig. 2). The same tendency was observed in all cultivation phases of transformants (Fig. 2). This phenomenon might be caused by galactose assimilation resulting in a

concentration decrease in the medium. Continued addition of galactose to the medium might achieve extended periods of induced expression.

In terms of industrial application, we also investigated the inducibility of the *GAL10* promoter in GY medium, a conventional nutrient-rich medium for *M. alpina* cultivation. The *GAL10* promoter was able to induce GUS activity when galactose was added into GY medium regardless of cultivation phase, as well as in the synthetic medium (Fig. 3). However, the induced GUS activity was lower and the induction response was slower than in synthetic medium (compare Fig. 2 and Fig. 3). This effect is likely caused by glucose in GY medium, because we observed that glucose slightly repressed GUS activity with the *GAL10* promoter in the presence of galactose (Table 2). In agreement with our findings, it has been reported for other microorganisms that glucose represses expression regulated by promoters that can be induced by carbon sources such as galactose and xylose (Götz 1995, Gancedo 1998, Johnston, et al. 1994, Meisenzahl, et al. 1997). In addition, the difference in nitrogen sources and trace elements between synthetic and complex media might also affect regulation and induction kinetics of this promoter. Further investigation of cultivation conditions could result in high levels of activity and/or prolonged induction with the *GAL10* promoter for potential use. Recently, functional lipids such as PUFAs have been recognized for their beneficial effects on

human health (Gill and Valivety 1997), and *M. alpina* has been utilized for the production of various PUFAs through molecular breeding (Jareonkitmongkol, et al. 1992, Jareonkitmongkol, et al. 1993, Kawashima, et al. 1997, Sakuradani, et al. 2013). The ability of the *GAL10* promoter that can be induced even in complex medium as well as in synthetic medium will be a great advantage for industrial lipid production by *M. alpina*.

The 5′ deletion analysis of the *GAL10* promoter region revealed that a relatively long length was required to regulate high GUS activity and response to the inducer (Fig. 4). This result suggests that transcription factor binding sites, enhancer elements and induction factor binding sites of the *GAL10* promoter are located in the far upstream region. A more detailed deletion analysis will lead to identification of functionally essential regulatory elements and elucidation of the inducible regulatory mechanisms of this promoter. Such information of inducible promoters is also useful for practical applications. For example, in *Saccharomyces cerevisiae*, the introduction of multiple copies of the consensus sequence, which is essential for the galactose-inducible promoters has been reported to improve inducibility of promoters (Giniger and Ptashne 1988).

M. alpina has been studied as one of model organisms for zygomycetes in molecular

biology. Zygomycetes, forming a class of fungi, have been widely used in the industrial fermentation field. For example, *Rhizomucor pusillus* has contributed to traditional cheese manufacturing as a producer of an alternative enzyme to the rennet (Arima, et al. 1970). Lipase from *Rhizopus oryzae* has been applied to biodiesel production (Kaieda, et al. 1999). There have been more many investigations of useful zygomycetes for production of industrially useful compounds (Millati, et al. 2005, Rodrigues and Fernandez-Lafuente 2010, Synowiecki and Al-Khateeb 2003). Many industrial applications using zygomycetes have been developed; however, basic molecular biological knowledge of zygomycetes is still limited, as compared with other microorganisms. The information which was obtained in this study might contribute to stimulate research for zygomycetes molecular biology.

In conclusion, we found two galactose-inducible promoters, *GAL1* and *GAL10* promoters in *M. alpina* 1S-4. The *GAL10* promoter is an inducible promoter with potential application in research and industry, because it can be sensitively induced depending on the presence/absence of an inducer in both synthetic and complex media. This is the first report characterizing inducible promoters of the zygomycetes oleaginous fungus, *Mortierella alpina*.

Acknowledgements

References

Amann E, Brosius J, Ptashne M (1983) Vectors bearing a hybrid trp-lac promoter useful for regulated expression of cloned genes in *Escherichia coli*. Gene 25: 167-178.

Ando A, Sakuradani E, Horinaka K, Ogawa J, Shimizu S (2009a) Transformation of an oleaginous zygomycete *Mortierella alpina* 1S-4 with the carboxin resistance gene conferred by mutation of the iron-sulfur subunit of succinate dehydrogenase. Curr Genet 55: 349-356.

Ando A, Sumida Y, Negoro H, Suroto DA, Ogawa J, Sakuradani E, Shimizu S (2009b) Establishment of *Agrobacterium tumefaciens*-mediated transformation of an oleaginous fungus, *Mortierella alpina* 1S-4, and its application for eicosapentaenoic acid producer breeding. Appl Environ Microbiol 75: 5529-5535.

Archer DB, Jeenes DJ, Mackenzie DA (1994) Strategies for improving heterologous protein production from filamentous fungi. Antonie van Leeuwenhoek 65: 245-250.

Arima K, Yu J, Iwasaki S (1970) Milk-clotting enzyme from *Mucor pusillus* var. Lindt. Methods Enzymol 19: 446-459.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:

248-254.

Cereghino JL, Cregg JM (2000) Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol Rev 24: 45-66.

Chien CT, Bartel PL, Sternglanz R, Fields S (1991) The two-hybrid system: A method to identify and clone genes for proteins that interact with a protein of interest. Proc Natl Acad Sci USA 88: 9578-9582.

Götz F (1995) A promoter-screening plasmid and xylose-inducible, glucose-repressible expression vectors for *Staphylococcus carnosus*. Gene 158: 91-96.

Gancedo JM (1998) Yeast carbon catabolite repression. Microbiol Mol Biol Rev 62: 334-361.

Gill I, Valivety R (1997) Polyunsaturated fatty acids, part 1: Occurrence, biological activities and applications. Trends Biotechnol 15: 401-409.

Giniger E, Ptashne M (1988) Cooperative DNA binding of the yeast transcriptional activator GAL4. Proc Natl Acad Sci USA 85: 382-386.

Giniger E, Varnum SM, Ptashne M (1985) Specific DNA binding of GAL4, a positive regulatory protein of yeast. Cell 40: 767-774.

Griggs DW, Johnston M (1991) Regulated expression of the *GAL4* activator gene in yeast provides a sensitive genetic switch for glucose repression. Proc Natl Acad Sci USA 88: 8597-8601.

Hansson L, Blackberg L, Edlund M, Lundberg L, Stromqvist M, Hernell O (1993) Recombinant human milk bile salt-stimulated lipase. Catalytic activity is retained in the absence of glycosylation and the unique proline-rich repeats. J Biol Chem 268: 26692-26698.

Ichishima E, Taya N, Ikeguchi M, Chiba Y, Nakamura M, Kawabata C, Inoue T, Takahashi K, Minetoki T, Ozeki K, Kumagai C, Gomi K, Yoshida T, Nakajima T (1999) Molecular and enzymic properties of recombinant 1,2-α-mannosidase from *Aspergillus saitoi* overexpressed in *Aspergillus oryzae* cells. Biochem J 339: 589-597.

Jareonkitmongkol S, Kawashima H, Shirasaka N, Shimizu S, Yamada H (1992) Production of dihomo-γ-linolenic acid by a Δ5-desaturase-defective mutant of *Mortierella alpina* 1S-4. Appl Environ Microbiol 58: 2196-2200.

Jareonkitmongkol S, Shimizu S, Yamada H (1993) Production of an eicosapentaenoic acid-containing oil by a Δ12 desaturase-defective mutant of *Mortierella alpina* 1S-4. J Am Oil Chem Soc 70: 119-123.

Jefferson RA, Burgess SM, Hirsh D (1986) ß-Glucuronidase from *Escherichia coli* as a gene-fusion marker. Proc Natl Acad Sci USA 83: 8447-8451.

Johnston M (1987) A model fungal gene regulatory mechanism: the *GAL* genes of *Saccharomyces cerevisiae*. Microbiol Rev 51: 458-476.

Johnston M, Davis RW (1984) Sequences that regulate the divergent *GAL1-GAL10* promoter in *Saccharomyces cerevisiae*. Mol Cell Biol 4: 1440-1448.

Johnston M, Flick J Spexton T (1994) Multiple mechanisms provide rapid and stringent glucose repression of *GAL* gene expression in *Saccharomyces cerevisiae*. Mol Cell Biol 14: 3834-3841.

Kaieda M, Samukawa T, Matsumoto T, Ban K, Kondo A, Shimada Y, Noda H, Nomoto F, Ohtsuka K, Izumoto E, Fukuda H (1999) Biodiesel fuel production from plant oil catalyzed by *Rhizopus oryzae* lipase in a water-containing system without an organic solvent. J Biosci Bioeng 88: 627-631.

Kawashima H, Kamada N, Sakuradani E, Jareonkitmongkol S, Akimoto K, Shimizu S (1997) Production of 8,11,14,17-cis-eicosatetraenoic acid by Δ5 desaturase-defective mutants of an arachidonic acid-producing fungus, *Mortierella alpina*. J Am Oil Chem Soc 74: 455-459.

MacKenzie DA, Jeenes DJ, Belshaw NJ, Archer DB (1993) Regulation of secreted protein production by filamentous fungi: Recent developments and perspectives. J Gen Microbiol 139: 2295-2307.

Meisenzahl AC, Shapiro L, Jenal U (1997) Isolation and characterization of a xylose-dependent promoter from *Caulobacter crescentus*. J Bacteriol 179: 592-600.

Millati R, Edebo L, Taherzadeh MJ (2005) Performance of *Rhizopus*, *Rhizomucor*, and *Mucor* in ethanol production from glucose, xylose, and wood hydrolyzates. Enzyme Microb Technol 36: 294-300.

Punt PJ, Van Biezen N, Conesa A, Albers A, Mangnus J, Van Den Hondel C (2002) Filamentous fungi as cell factories for heterologous protein production. Trends Biotechnol 20: 200-206.

Rodrigues RC, Fernandez-Lafuente R (2010) Lipase from *Rhizomucor miehei* as an industrial biocatalyst in chemical process. Journal of Molecular Catalysis B: Enzymatic 64: 1-22.

Sakuradani E, Ando A, Shimizu S, Ogawa J (2013) Metabolic engineering for the production of polyunsaturated fatty acids by oleaginous fungus *Mortierella alpina* 1S-4. J Biosci Bioeng 116: 417-422.

Sakuradani E, Kobayashi M, Shimizu S (1999) Δ^9-Fatty acid desaturase from arachidonic acid-producing fungus. Unique gene sequence and its heterologous expression in a fungus, *Aspergillus*. Eur J Biochem 260: 208-216.

Shen WJ, Forde BG (1989) Efficient transformation of *Agrobacterium* spp. by high voltage electroporation. Nucleic Acids Res 17: 8385.

Shimizu S, Ogawa J, Kataoka M, Kobayashi M (1997) Screening of novel microbial enzymes

for the production of biologically and chemically useful compounds. Adv Biochem Eng 58: 45-87.

Sumi A, Okuyama K, Kobayashi K, Ohtani W, Ohmura T, Yokoyama K (1999) Purification of Recombinant Human Serum Albumin. Efficient purification using STREAMLINE. Bioseparation 8: 195-200.

Synowiecki J, Al-Khateeb NA (2003) Production, Properties, and Some New Applications of Chitin and Its Derivatives. Crit Rev Food Sci Nutr 43: 145-171.

Takeno S, Sakuradani E, Murata S, Inohara-Ochiai M, Kawashima H, Ashikari T, Shimizu S (2004a) Cloning and sequencing of the *ura3* and *ura5* genes, and isolation and characterization of uracil auxotrophs of the fungus *Mortierella alpina* 1S-4. Biosci, Biotechnol, Biochem 68: 277-285.

Takeno S, Sakuradani E, Murata S, Inohara-Ochiai M, Kawashima H, Ashikari T, Shimizu S (2004b) Establishment of an overall transformation system for an oil-producing filamentous fungus, *Mortierella alpina* 1S-4. Appl Microbiol Biotechnol 65: 419-425.

Takeno S, Sakuradani E, Tomi A, Inohara-Ochiai M, Kawashima H, Shimizu S (2005) Transformation of oil-producing fungus, *Mortierella alpina* 1S-4, using Zeocin, and application to arachidonic acid production. J Biosci Bioeng 100: 617-622.

Terpe K (2006) Overview of bacterial expression systems for heterologous protein production: From molecular and biochemical fundamentals to commercial systems. Appl Microbiol Biotechnol 72: 211-222.

Wickes BL, Edman JC (1995) The *Cryptococcus neoformans GAL7* gene and its use as an inducible promoter. Mol Microbiol 16: 1099-1109.

**Table 1.** PCR primers used to clone the *GAL1* and *GAL10* promoter regions

Primer name	Sequence (5′ to 3′)
GAL1pF	AATA<u>TCTAGA</u>[a]ACCACGCATGACAATGCCAC
GAL1pR	AAGA<u>ACTAGT</u>TGTAAAAGGGGCTGACAGTG
GAL10pF	AATA<u>TCTAGA</u>GGTTCCGAGAGGTGGATTTG
GAL10pR	ATAA<u>TCTAGA</u>TGGCTCCTGAAAGGACGAG
GAL10p2000F	AAT<u>TCTAGA</u>CGCAGAGTGATGGTCATTACC
GAL10p1600F	AAT<u>TCTAGA</u>CTCTATGGCAAGATTACGAG
GAL10p1200F	AAT<u>TCTAGA</u>TGCTCGTGAAGAGGGGCAC
GAL10p800F	ACG<u>TCTAGA</u>CATTTTTTGCCGCCAATTCTG
GAL10p400F	ATT<u>TCTAGA</u>CCCCCGCCTATTTTTTTTTTC

[a] The underlined sequences indicate inserted XbaI (TCTAGA) and SpeI (ACTAGT) sites.

Table 2. GUS activity resulting from the β-glucuronidase gene fused to *GAL1* and *GAL10* promoters in transformants cultivated on solid media containing different carbon sources

| Carbon source	GUS activity [nmol/(mg·min)]	
	GAL1 p	*GAL10* p
no carbon source	593.8 ± 43.4	72.1 ± 14.9
glucose	440.1 ± 46.4	19.5 ± 2.3
galactose	3360.1 ± 780.7	1890.8 ± 372.1
lactose	1653.6 ± 84.2	282.6 ± 67.9
raffinose	916.7 ± 63.0	63.3 ± 14.6
glucose + galactose	3407.3 ± 253.6	1562.6 ± 137.2
lactose + galactose	3543.4 ± 526.7	1876.8 ± 299.2
raffinose + galactose	3152.3 ± 187.9	2223.9 ± 256.9

All transformants were cultivated in SC medium containing 2% of each sugar substituted for glucose for 3 days at 28°C. The values represent mean GUS activity of three transformant lines (± standard deviation).
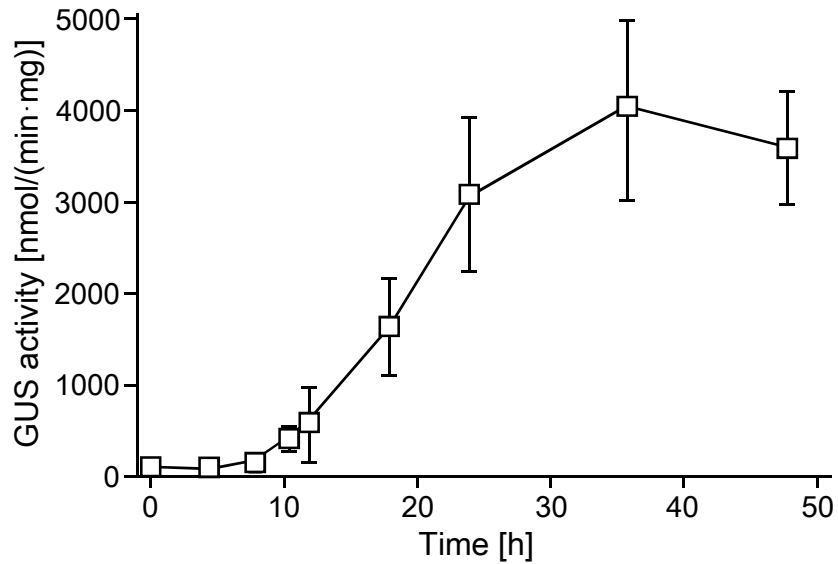
Fig. 1. GUS activity in response to *GAL10* promoter induction by galactose addition in submerged cultivation. Transformants were pre-cultivated in SC liquid medium containing raffinose substituted for glucose for 4 days, and then galactose was added at t=0. GUS activity was monitored over a 48 h time course. The values represent mean GUS activity of three transformant lines (± standard deviation).
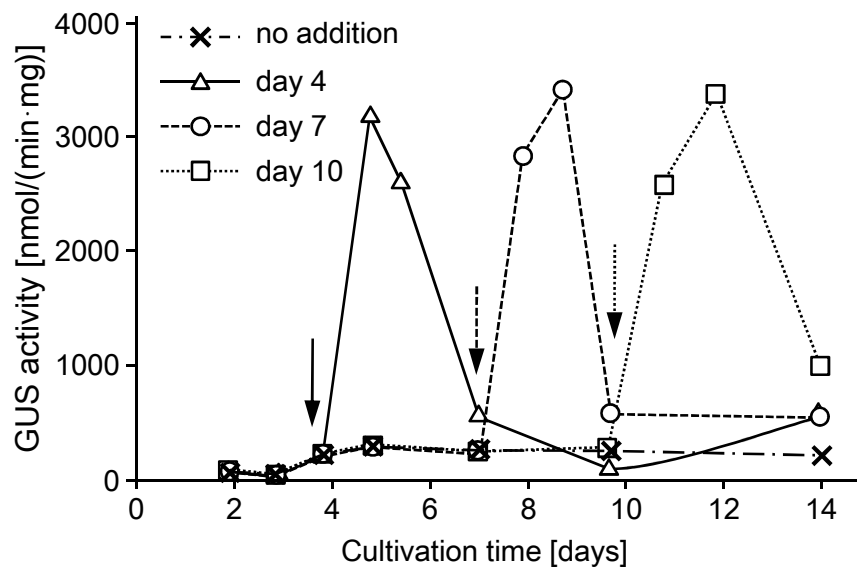
Fig. 2. GUS activity in response to *GAL10* promoter induction by galactose addition in different cultivation phase in synthetic medium. Transformants were cultivated in SC liquid medium containing raffinose substituted for glucose, and then galactose was added on day 4, 7 or 10 (arrows). The values represent mean GUS activity of three transformant lines.
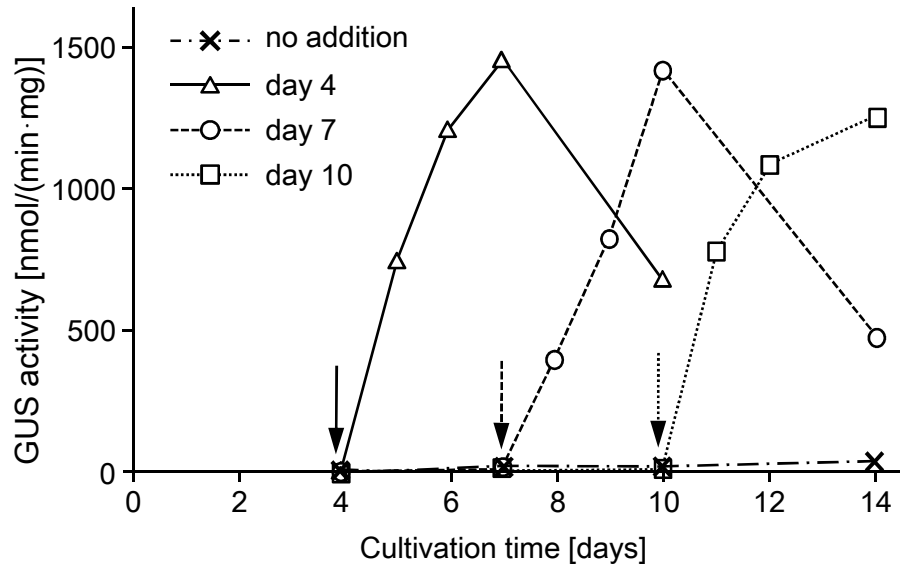
Fig. 3. GUS activity in response to *GAL10* promoter induction by galactose addition in different cultivation phases in complex medium. Transformants were cultivated in GY liquid medium, and galactose was added on day 4, 7 or 10 (arrows). The values represent mean GUS activity of three transformant lines.
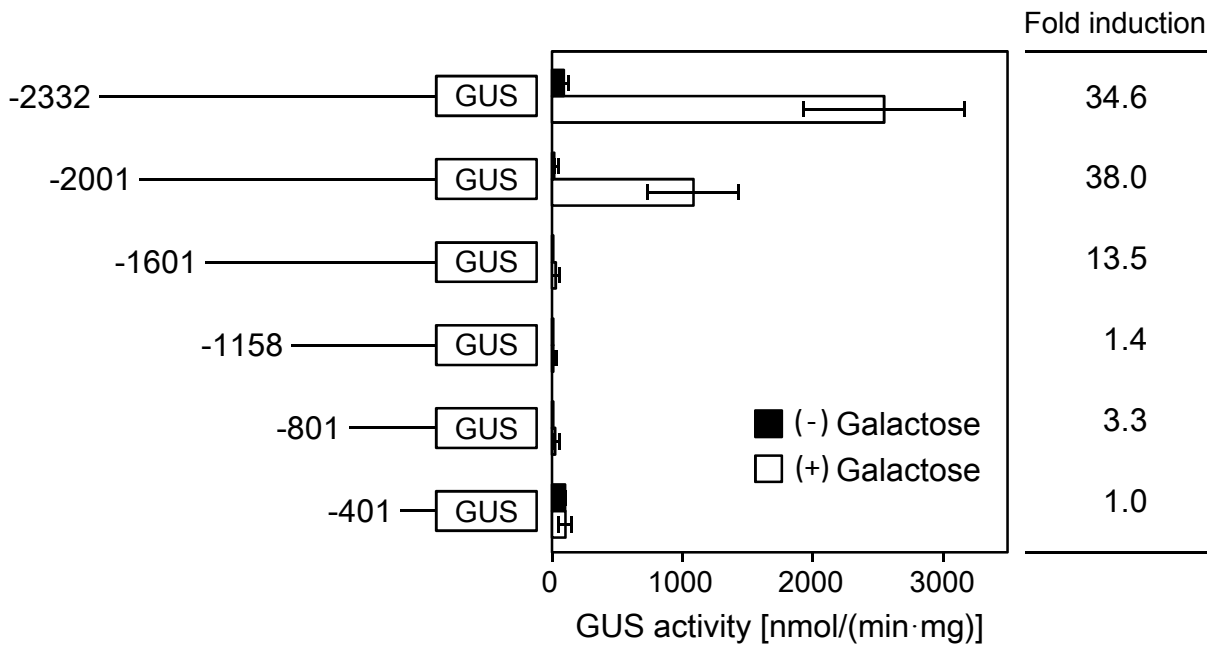
Fig. 4. 5′-deletion analysis of the *GAL10* promoter. Left column) Constructs with different 5′ upstream deletions of the promoter region are shown. For each construct, the length of the fragment upstream from the transcription start site is shown on the left end. Center column) GUS activity levels with the deleted constructs in *M. alpina* transformants are shown. All transformants were precultivated for 4 days in SC liquid meduim containing raffinose substituted for glucose, added (+) or not added (-) with galactose, and then cultivated for a day. Bars represent the mean values with standard deviations of GUS activity in 10 individual transformant lines for each construct. Right column) Fold induction of the each deleted constructs (with (+)/without (-) galactose) in *M. alpina* transformants are shown.