Selection and characterization of promoters based on genomic approach for the molecular breeding of oleaginous fungus *Mortierella alpina* 1S-4

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Abstract

To express a foreign gene effectively, a good expression system is required. In this study, we investigated various promoters as useful tools for gene manipulation in oleaginous fungus *Mortierella alpina* 1S-4. We selected and cloned the promoter regions of 28 genes in *M. alpina* 1S-4 on the basis of expression sequence tag (EST) abundance data. The activity of each promoter was evaluated by using the *β*-glucuronidase (*GUS*) reporter gene. Eight of these promoters were shown to enhance GUS expression more efficiently than a histone promoter, which is conventionally used for the gene manipulation in *M. alpina*. Especially, the predicted protein 3 (*PP3*) and the predicted protein 6 (*PP6*) promoters demonstrated approximately 5-fold higher activity than the histone promoter. The activity of some promoters changed along with the cultivation phase of *M. alpina* 1S-4. Seven promoters with constitutive or time-dependent, high-level expression activity were selected, and deletion analysis was carried out to determine the promoter regions required to retain activity. This is the first report of comprehensive promoter analysis based on a genomic approach for *M. alpina*. The promoters described here will be useful tools for gene manipulation in this strain.

Key Words: *Mortierella alpina*, promoter, Expression sequence tag, gene manipulation

Introduction

The properties of promoters strongly influence the expression level and duration of target genes (Jefferson, et al. 1987, Mumberg, et al. 1994, Wurm 2004). The application of highly expressing and/or regulated promoters is one of most important factors in a valuable expression system. Many investigations for useful promoters have been carried out and contributed to the improvement of heterologous protein production in various microorganisms. In *Escherichia coli*, for example, the bacteriophage T7 promoter system has been used to accumulate recombinant proteins at high concentrations (40–50%) of total cell protein (Baneyx 1999). In the methylotrophic yeast *Pichia pastoris*, strong methanol-inducible promoters have been used to produce various medically important proteins have been produced (Cereghino and Cregg 2000, Hansson, et al. 1993, Sumi, et al. 1999). Also in fungi, mainly in *Aspergillus* species, the investigation and modification of high-expression promoters has led to successful high-level production of heterologous proteins such as glucoamylase, protease, and lipase (Archer, et al. 1994, Ichishima, et al. 1999, MacKenzie, et al. 1993, Punt, et al. 2002).

Recently, functional lipids such as polyunsaturated fatty acids (PUFAs) have been recognized for their beneficial effects on human health (Gill and Valivety 1997). In

addition, lipid fermentation by microorganisms with high fatty acid selectivity is expected to serve as an alternative method supplying PUFA more stably than conventional production from plant-seed oils and fish oils (Ratledge 1993). Therefore, the development of gene manipulation tools for lipid-producing microorganisms is important. In fact, various lipids have been produced by means of molecular breeding of microorganisms in some studies (Beopoulos, et al. 2011, Courchesne, et al. 2009, Raghukumar 2008, Sakuradani, et al. 2013). *Mortierella alpina* 1S-4, an oleaginous fungus, is a lipid-producing microbe (Shimizu, et al. 1997). To date, the production of various kinds of PUFAs, such as arachidonic acid, dihomo-γ-linolenic acid, Mead acid and eicosapentaenoic acid has been achieved by molecular breeding of *M. alpina* (Jareonkitmongkol, et al. 1992, Jareonkitmongkol, et al. 1993, Kawashima, et al. 1997, Sakuradani, et al. 2013). Basic molecular breeding tools such as gene delivery systems, host-vector systems and transformation systems using auxotrophy or antibiotic resistance have been established in *M. alpina* 1S-4 (Ando, et al. 2009a, Takeno, et al. 2004a, Takeno, et al. 2005). However, the gene modifiability of *M. alpina* is still limited due to lack of identification of variations in promoters (Mackenzie, et al. 2000). For further development of gene expression systems, such as multiple gene expression, temporally regulated expression and inducible expression, it is necessary to prepare

various kinds of promoters. Enrichment of promoter types would contribute to improving PUFA productivity and modifying PUFA composition, and may help elucidate the mechanisms regulating gene expression in *M. alpina*.

In general, promoter discovery in fungal biotechnology has been mainly based on the information of highly- or constitutively-expressed proteins (Hata, et al. 1991, Tada, et al. 1991). Recently, expression sequence tag (EST) analysis has been used as a powerful tool for investigating expressed genes. EST abundance data can present directly gene transcriptional levels, and make possible widespread approaches to find desired promoters in combination with the genomic information (Kusakabe, et al. 1994, Ranamalie Amarasinghe, et al. 2006).

In this study, we selected and cloned promoter regions of various genes of *M. alpina* 1S-4 on the basis of EST abundance data, and characterized these promoter regions by fusing β-glucuronidase (GUS) reporter assays.

Materials and Methods

Strains, media, and growth conditions

A uracil auxotroph (*ura5*$^-$ strain), previously isolated from *M. alpina* 1S-4 deposited at 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 agar 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 for reporter assays and extracting genomic DNA. GS medium (5% [wt/wt] soy flour, 0.3% K_2HPO_4, 0.05% $MgCl_2 \cdot 6H_2O$ and 0.05% $CaCl_2 \cdot 2H_2O$) was used for large-scale cultivation. Liquid cultivations were performed at 28°C with shaking (300 rpm), except for large-scale cultivation when a jar-fermentor was used.

Escherichia 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 the transformation, cultivation and infection of *A. tumefaciens*, respectively. The compositions of LB-Mg agar medium, MM, and IM have been described previously (Takeno, et al. 2004b).

Genomic DNA preparation

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

Construction of cDNA libraries of M. alpina *1S-4 and EST analysis*

For large-scale cultivation, an inoculum was prepared in a 50-L jar fermentor containing 30 L of GY medium supplemented with 0.1% soybean oil, followed by cultivation for 2 d at 28°C. The main cultivation was carried out in a 10-kL fermentor (Kansai Chemical Engineering Co., Hyogo, Japan) with 4 kL of GS medium at 26°C with stirring. At 18, 42, 66, 90 and 114 h after starting cultivation, 5.33% or 4% glucose was added. For extracting the total RNA of *M. alpina*, fungal mycelia were sampled after 17, 25, 42, 114, 209 and 281 h of cultivation. Total RNA was extracted from each

sample by using RNeasy Mini Kit (QIAGEN).

First strand cDNA was synthesized by using SOLiD^{TM} Total RNA-Seq for Whole Transcriptome Libraries (Applied Biosystems, Inc., California, USA). For EST and transcriptome analysis, we used a research contract service (Genaris, Inc., Kanagawa, Japan).

Cloning of M. alpina *promoters*

Information regarding selected promoters analyzed in this study is shown in Table 1. Selected promoter regions were cloned from the genome of *M. alpina* 1S-4 by PCR performed using specific primers (Table S1) designed on the basis of the information available in the genomic database for this strain. Putative PolII transcriptions sites in these promoter sequences were predicted by using Promoter 2.0 Prediction Server (http://www.cbs.dtu.dk/services/Promoter/). For deletion constructs, the anti-sense primers used for PCR are shown in Table S1 and forward primers are shown in Table S2. *Xba*I and *Spe*I restriction enzyme sites were created at the 5′ end of each forward primer and at the 3′ end of each reverse primer, respectively. When an *Xba*I site was present in the promoter region, an *Spe*I site was created instead of the *Xba*I site at the 5′ end of the forward primer. When an *Spe*I site was present in the promoter region, an *Xba*I site was

created instead of the *Spe*I site at the 3′ end of the reverse primer.

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 the *ura5* marker gene (Takeno, et al. 2004a) were amplified from the genomic DNA of *M. alpina* 1S-4. The *ura5* expression cassette controlled by a histone promoter and *SdhB* terminator was generated by fusion PCR with additional *EcoR*I and *Xba*I restriction enzyme sites at the 5′ and 3′ ends, respectively, of this cassette. The *ura5* expression cassette, digested with *EcoR*I and *Xba*I, was ligated to pBIG3ura5s (Ando, et al. 2009b) 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 *BamH*I restriction enzyme sites at the 5′ and 3′ flanking ORFs, respectively. The *GUS* expression cassette, controlled by a histone promoter and *SdhB* terminator, was generated by fusion PCR with additional *Xba*I and *Nhe*I restriction sites at the 5′ and 3′ ends of the cassette,

respectively. This *GUS* expression cassette was digested with *Xba*I and *Nhe*I and ligated to pBIG35Zh digested with same restriction enzymes and designated pBIG35ZhGUSm (Fig. 1). In this vector, the histone promoter region, located upstream of the *GUS* gene, can be removed by digestion with *Xba*I and *Spe*I, and replaced by another promoter fragment digested with *Xba*I and/or *Spe*I for promoter assays.

Transformation of M. alpina *1S-4 ura5*$^{-}$ *strain*

A spore suspension of *M. alpina* 1S-4 *ura5*$^{-}$ strain was freshly prepared by harvesting from cultures grown on Czapek-Dox agar medium supplemented with 0.05 mg/ml uracil and then filtering the suspension through Miracloth (Calbiochem) (Takeno, et al. 2004b).

Transformation of *M. alpina* 1S-4 *ura5*$^{-}$ strain was performed using the *Agrobacterium tumefaciens*-mediated transformation (ATMT) method described previously (Ando, et al. 2009b) with slight modification. Briefly, *Agrobacterium 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). *Agrobacterium 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 (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 a spore suspension (10^8 spores/ml) of *M. alpina* 1S-4 *ura5*$^-$ strain, and then spread on membranes (Whatman #50 Hardened Circles, 70 mm, Whatman International Ltd. UK) kept on cocultivation media (IM with 1.5% agar) and incubated at 23°C for 5 d. After cocultivation, the membranes were transferred to uracil-free SC agar plates that contained 0.03% Nile blue A (Sigma-Aldrich Japan) to distinguish between fungal colonies and the white color of the membrane. After 2 d of incubation at 28°C, hyphae from visible fungal colonies were transferred to fresh uracil-free SC agar plates, this was repeated 3 times to obtain candidates. Integration of the vector into the chromosome of the host strain was verified by PCR, as described previously (Takeno, et al. 2004b).

Preparation of cell-free extracts for GUS assays

Cell-free extracts of *M. alpina* were prepared by a slight modification of a method

described previously (Takeno, et al. 2004a). All transformants and the wild-type strain of *M. alpina* 1S-4 were cultivated in 10 ml of GY medium for 2–14 d at 28°C with shaking (300 rpm), harvested by suction filtration, and washed twice with sterile water. Fungal mycelia were suspended in 2 volumes of 100 mM Tris-HCl containing 5 mM 2-mercaptoethanol (pH 7.5) and then disrupted by using a bead shocker (Wakenyaku Co. Ltd., Kyoto, Japan) at 5,000 rpm for 30 s twice 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

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

Nucleotide sequence accession number

The sequences of selected promoters were deposited in DDBJ, under the following accession numbers; PP1 (AB871464), PP2 (AB859208), PP3 (AB859209), SSA2 (AB859212), PP7 (AB859213), SSA22 (AB871467), PP4 (AB871465), PP8 (AB871479), SAH1 (AB871466), PET9 (AB871478), HSP104 (AB871471), HSC82 (AB859211), UBC5 (AB871477), CDA1 (AB871469), RPP0 (AB871473), PP5 (AB871472), PP6 (AB859210), RPS16B (AB871474), EFB1 (AB871460), TDH1 (AB871475), CIT1 (AB859214.), TIF2 (AB871476), CAT2 (AB871468), ELO1 (AB871470), IPP1 (AB871461), OLE1 (AB871462) and PGK1 promoter (AB871463).

Results

Selection, cloning and evaluation of various promoters of M. alpina *1S-4*

We cultivated *M. alpina* 1S-4 on GS medium and prepared cDNA libraries by using RNA extracted from the mycelia on different cultivation stages (see Materials and Methods). EST analysis were performed for each cDNA sample, and the abundances of each EST clone during all cultivation stages were summed (data not shown). These totals were sorted in descending order. On the basis of these EST abundance data and previous reports regarding conventional promoters of other organisms (Makrides 1996, Ostergaard, et al. 2000, Twyman, et al. 2003, Wurm 2004), putative promoter regions of 28 genes of *M. alpina* 1S-4 were selected as candidates of highly-expressing and/or temporally-regulated promoters (Table 1). Considering of the positions of putative transcriptional factor-binding sites in each selected promoter region, we cloned approximately 1000–2500 bp of the 5′ flanking region of individual ORFs were cloned as putative promoter regions from the genomic DNA of *M. alpina* 1S-4. To evaluate the activity of these putative promoters in *M. alpina*, pBIG35ZhGUSm plasmids carrying each putative promoter region, instead of the histone promoter, located upstream of the *β*-glucuronidase (*GUS*) gene were constructed (Fig. 1) and transformed into *M. alpina*

1S-4 using the ATMT method. For each construct, 30 transformants were randomly selected and cultivated for 5 d in GY liquid medium, and then their GUS activities were measured. Due to the variety in GUS activity in individual *M. alpina* transformant lines with each promoter construct (a representative pattern is shown in Fig. 2), we used the average value of GUS activities in the 10 moderately expressing lines for comparison with different promoter activities (Fig. 3). As shown in Fig. 3, *PP1*, *PP3, SSA2*, *PP7*, *HSC82*, *PP6*, *TDH1* and *CIT1* promoters led to increased GUS activity compared with a conventional histone promoter. In particular, *PP3* and *PP6* promoters showed approximately 5-fold higher activity than the histone promoter.

We also carried out the same experiments with GS medium, which was used for large-scale cultivation (see Materials and Methods). There were no apparent differences in the GUS activity levels between GY and GS media (data not shown). Therefore, GY medium was used to cultivate transformants in all subsequent GUS assays.

Time course measurements of promoter activity during cultivation of M. alpina *1S-4*

Transformants with each promoter construct were cultivated in GY medium for 2–14 d and then GUS activity was evaluated in order to investigate the effect of cultivation time on GUS activity with different promoters (Fig. 4). Based on the pattern of

time-dependent changes in GUS activity, promoters could be categorized into the following 4 groups; GUS activity levels controlled by the *HSC82*, *PP7*, *SSA2*, *HSP104*, *UBC5* or *PET9* promoter were almost constant throughout the cultivation period (Fig. 4A). With the *CIT1*, *PP8*, *SAH1*, *EFB1*, *OLE1*, *HSC82*, *CDA1*, *RPP0*, *RPS16B* or *CAT2* promoter, GUS activity levels were higher in the early stage of cultivation and then decreased (Fig. 4B). GUS activity controlled by the *PP6*, *ELO1* or *TDH1* promoter peaked at the middle stage of cultivation (Fig. 4C). With the *PP3*, *PP2*, *PP4*, *PP5*, *SSA22*, *IPP1* or *PGK1* promoter, GUS activity levels were low in the early stage, and then increased with cultivation time (Fig. 4D).

PP2, *PP3*, *PP6*, *PP7*, *SSA2*, *HSC82* and *CIT1* promoters with constitutive or time-dependent high-level activity were selected and used for subsequent studies.

Deletion analysis

In order to investigate the length of the promoter regions required to maintain high expression activity, a series of 5′ deletion constructs of the 7 selected promoters were generated (Fig. 5, left column) and introduced into *M. alpina* 1S-4. For each deletion construct, 30 randomly selected transformants were cultivated in GY medium for the appropriate number of days based on the above results, and then GUS activity was

evaluated. For comparison, the GUS activity levels of 10 moderately expressing lines were averaged and represented as a value relative to each full-length promoter, which was set as 100% (Fig. 5, right column). In the *PP2*, *PP3* and *PP6* promoters, relatively long lengths of the promoter regions (over 1,000 bp) were required for high GUS expression, and the GUS activity levels dramatically diminished with deletion of the 5′ regions. In contrast, the other promoters maintained high activity even in relatively short regions (approximately 400–800 bp).

Discussion

In general, promoters that are useful for gene manipulation systems exhibit either constitutively high, time-dependent and/or conditionally inducible expression. Thus, we investigated and screened beneficial promoters for *M. alpina* gene manipulation using an EST-based approach in this view point. EST abundance data can provide gene expression levels without post-translational influence. Therefore, by using an EST-based approach, the desired promoters can be identified more directly and efficiently than by using conventional approaches based on information on protein expression.

In many cases, EST analysis is employed to obtain transcriptional information at a certain point in the cultivation period. Because the transcriptional level of each gene generally changes depending on the cultivation stage, we carried out EST analysis with *M. alpina* at different cultivation stages. On the basis of the EST data and previous reports on conventional promoters of other organisms, 28 promoters of *M. alpina* 1S-4 were selected as candidates for highly expressing and/or regulated promoters (see Table 1).

The *GUS* reporter gene was used to monitor the promoter activity in this study because the *GUS* gene has been commonly used as a reporter gene for promoter assays for various organisms (Jefferson, et al. 1987, Tada, et al. 1991). In addition, we considered

that this study also means investigation of heterologous gene expression in *M. alpina*, because the *GUS* gene is a heterologous gene for this strain.

The GUS activity in *M. alpina* transformant lines with each promoter construct was distributed across a wide range (Fig. 2). This dispersion might be attributable to the differing locations of the GUS gene in chromosomal DNA, *i.e.*, the position effect. It has previously been reported that *M. alpina* transformants generated by the ATMT method have a single copy of T-DNA at a random location in chromosomal DNA (Ando, et al. 2009b).

Our comprehensive analysis showed that the *PP3* and *PP6* promoters were demonstrate remarkably higher GUS activity than the conventional histone promoter in *M. alpina*. The functions of the proteins coded by the *PP3* and *PP6* genes are unknown. Investigation of the function of these proteins functions might lead to new findings, which may in turn lead to new insights on *M. alpina* physiology. Interestingly, the GUS expression levels were not necessarily proportional to the EST abundance values (compare Fig. 3 with Table 1). There were some cases where the GUS expression levels were much lower than expected from the EST abundance data, *e.g.* the *SSA22* and *PP8* promoters. In such cases, other factors besides promoters, such as the terminator and post-transcriptional processing might lead to high-transcriptional levels of the original

gene, unlike the findings seen for heterologous GUS gene expression.

Time-course measurements of GUS activity levels with various promoters showed several temporally-different patterns of expression (Fig. 4). Also in this examination, the GUS expression patterns were not necessarily consistent to the time course of EST abundance values (data not shown). It might be caused by difference of cultivation conditions. Especially, we performed glucose feeding on industrial cultivation for EST analysis (see Materials and Methods). Glucose starvation might be one of most important factors for cultivation phase-specific expression.

To our knowledge, this is the first report of time-dependent expression promoters in *M. alpina*. These promoters allow for phase-specific expression in *M. alpina*, unlike the conventional histone promoter expressing constitutively during cultivation time (data not shown). These time-dependent promoters could contribute to more efficient production of PUFAs in *M. alpina* by means of temporal coordination of enzyme expression with PUFA biosynthesis.

For the 5′ deletion analysis of promoter regions, 7 promoters were selected because of their characteristic expression patterns, such as high-level expression and/or time-dependent expression. A relatively long length (over 1,000 bp) was required to maintain high activity in the *PP2*, *PP3* and *PP6* promoters. This finding is agreement

with the prediction of putative transcription factor binding sites of these promoters (Fig. 5). In contrast, the *SSA2*, *PP7*, *HSC82* and *CIT1* promoters retained sufficient activity even in the truncated form (400–800 bp). These short promoters with high activity will be advantageous in applications involving *M. alpina* gene manipulation because they will be useful for convenient vector construction.

In some promoters, *SSA2*p and *HSC*82p, the length of promoter region with high activity were not correlated with the position of putative transcription factor binding sites (Fig. 5). Practically the reliable prediction for such sites is difficult in zygomycetes including *M. alpina* because the investigation of their transcriptional regulatory mechanism is still underdeveloped. More detailed deletion analysis and consensus sequence analysis of highly-expressing and/or regulated promoters will help identify functionally essential elements for transcriptional regulation. This in turn could help elucidate the transcriptional regulatory mechanisms of *M. alpina*. The information of transcriptional regulatory elements of promoters for high-level expression and time-dependent expression is also useful for applications. For example, in *Aspergillus oryzae*, the introduction of multiple copies of the consensus sequence found in the high-expression promoters has been reported to improve promoter activity (Minetoki, et al. 1998). We could not find such consensus sequences inherent in each expression

property of promoters identified in this study (data not shown), therefore more detailed investigations are required.

In conclusion, we found several potent promoters with constitutive or time-dependent high-expression activity by means of an EST-based approach in *M. alpina* 1S-4. Furthermore, the lengths of these promoter regions required to retain high expression levels were estimated by deletion analysis. The promoters data generated in this study will be beneficial for improvement in PUFA productivity and modification of PUFA composition, and may help elucidate the regulatory mechanisms of gene expression in *M. alpina*.

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Table 1. Information regarding genes for selected promoters

Gene	Annotation	Relative EST transcript abundance[a]
PP1	Predicted protein	35.7
PP2	Predicted protein	29.0
PP3	Predicted protein	11.7
SSA2	ATP binding protein (member of HSP70 family)	8.9
PP7	Predicted protein	7.9
SSA22	ATP binding protein (member of HSP70 family)	7.6
PP4	Predicted protein	7.3
PP8	Predicted protein	6.6
SAH1	*S*-Adenosyl-L-homocysteine hydrolase	6.6
PET9	ADP/ATP carrier of the mitochondrial inner membrane	6.0
HSP104	Hsp that cooperates with Hsp40 and Hsp70	5.9
HSC82	Cytoplasmic chaperone of the Hsp90 family	5.6
UBC5	Ubiquitin-conjugating enzyme	4.7
CDA1	Chitin deacetylase	4.5
RPP0	Ribosomal protein P0	4.0
PP5	Predicted protein	4.0
PP6	Predicted protein	3.8
RPS16B	Protein component of 40S ribosormal subunit	3.2
EFB1	Translation elongation factor 1 beta	2.6
TDH1	Glyceraldehyde-3-phosphate dehydrogenase	2.4
CIT1	Citrate synthase	2.0
TIF2	Translation initiation factor eIF4A	1.9
CAT2	Carnitine acyl-CoA transferase	0.9
ELO1	Fatty acid elongase I	0.7
IPP1	Cytoplasmic inorganic pyrophosphatase	0.7
OLE1	Delta-9 fatty acid desaturase	0.6
PGK1	3-Phosphoglycerate kinase	0.4

[a]EST abundance data show the total for EST transcriptional abundance at different cultivation stages, by using relative values for histone H4.1. For cultivation conditions, see materials and methods.

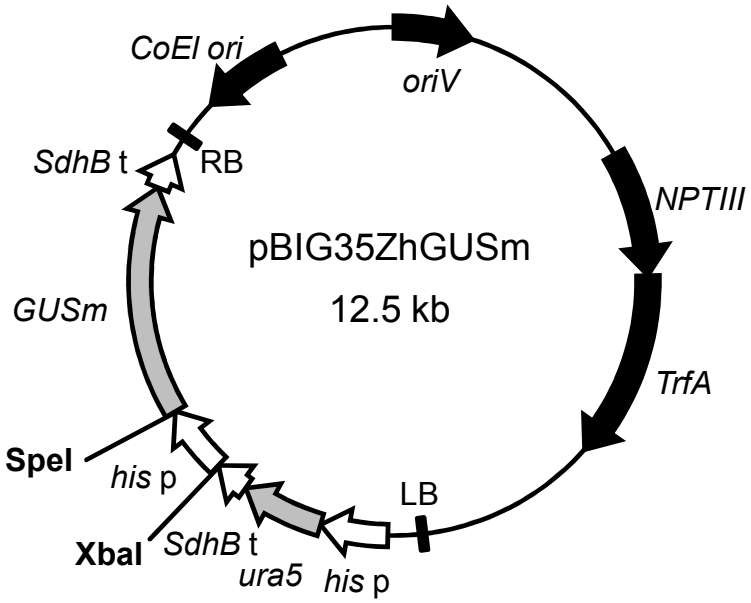


Fig. 1. Vector construct used in *M. alpina* 1S-4 promoter assays. *GUSm*, codon-optimized *β*-glucuronidase gene for *M. alpina*; *his* p, *M. alpina* histone H4.1 promoter short fragment; *SdhB* t, *M. alpina* SdhB transcription terminator; *ura5,* orotate phosphoribosyl transferase gene of *M. alpina* 1S-4; *NPTIII*, neomycin phosphotransferase III gene; *TrfA,* TrfA locus, which produces 2 proteins that promote replication of the plasmid; *ColEI ori*, ColEI origin of replication; *oriV,* pRK2 origin of replication; RB, right border; LB, left border.

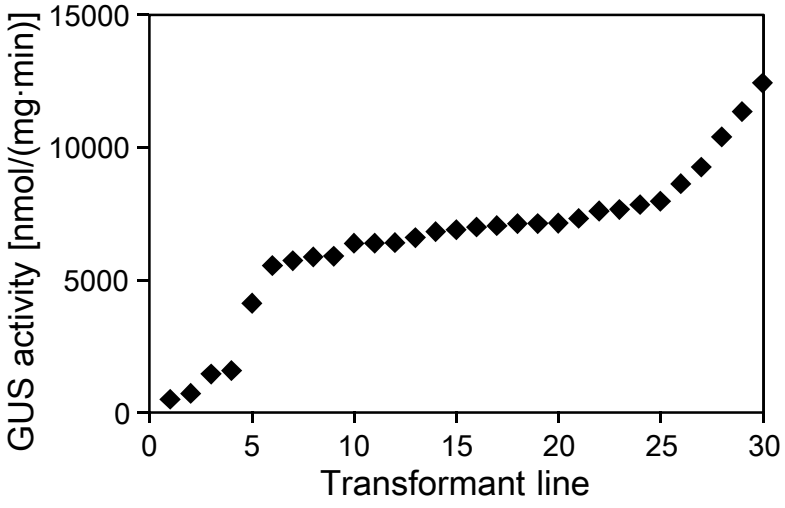


Fig. 2. Distribution of GUS activity levels driven by the *HSC82* promoter in *M. alpina* transformants cultivated for 5 d in GY liquid medium. Each plot denotes individual transformants, and all plots are sorted in ascending order of GUS activity. GUS activity is expressed in nanomoles of *p*-nitrophenol produced per minute per milligram of protein.

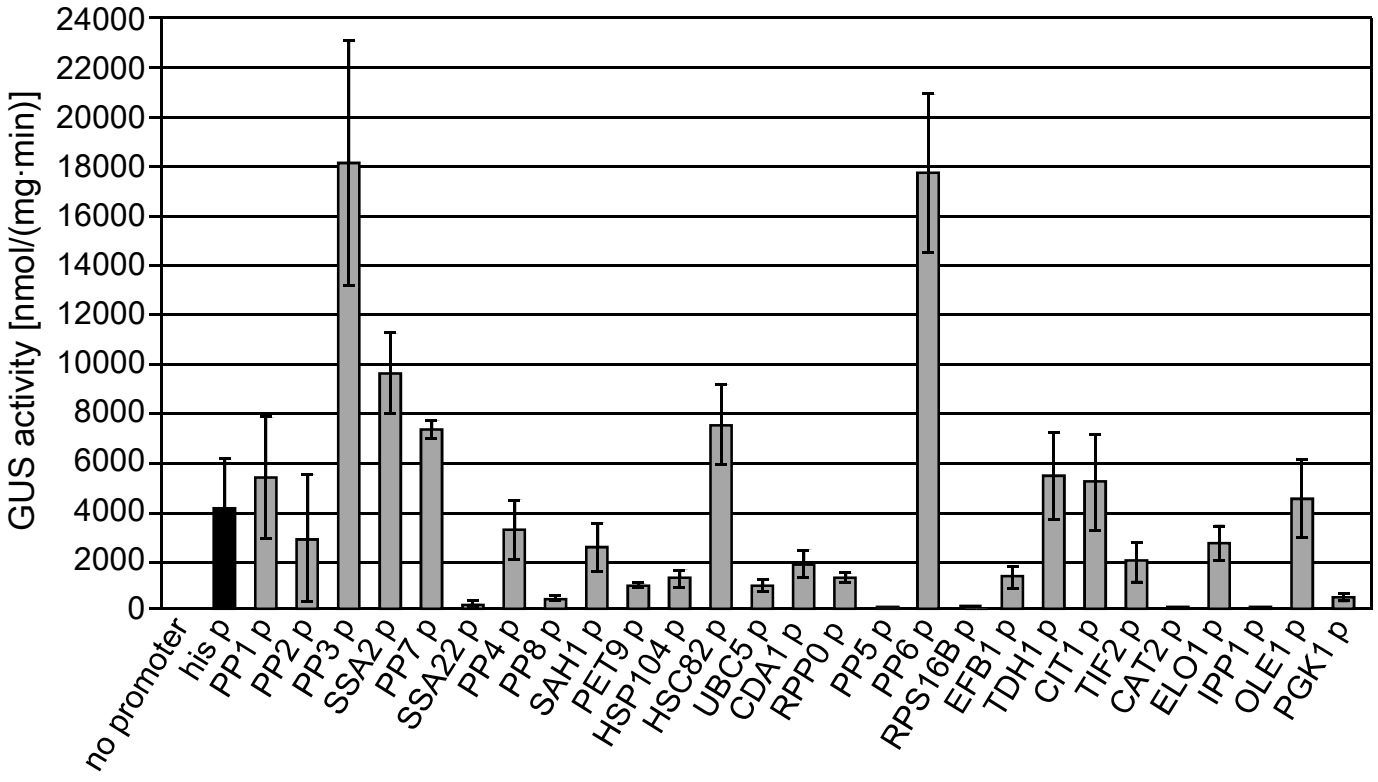


Fig. 3. GUS activity driven by various promoters in *M. alpina* transformants cultivated for 5 d in GY liquid medium. GUS activity is expressed in nanomoles of *p*-nitrophenol produced per minute per milligram of protein. The Bars represent the mean values with standard deviations of GUS activity in 10 individual transformant lines for each promoter construct.

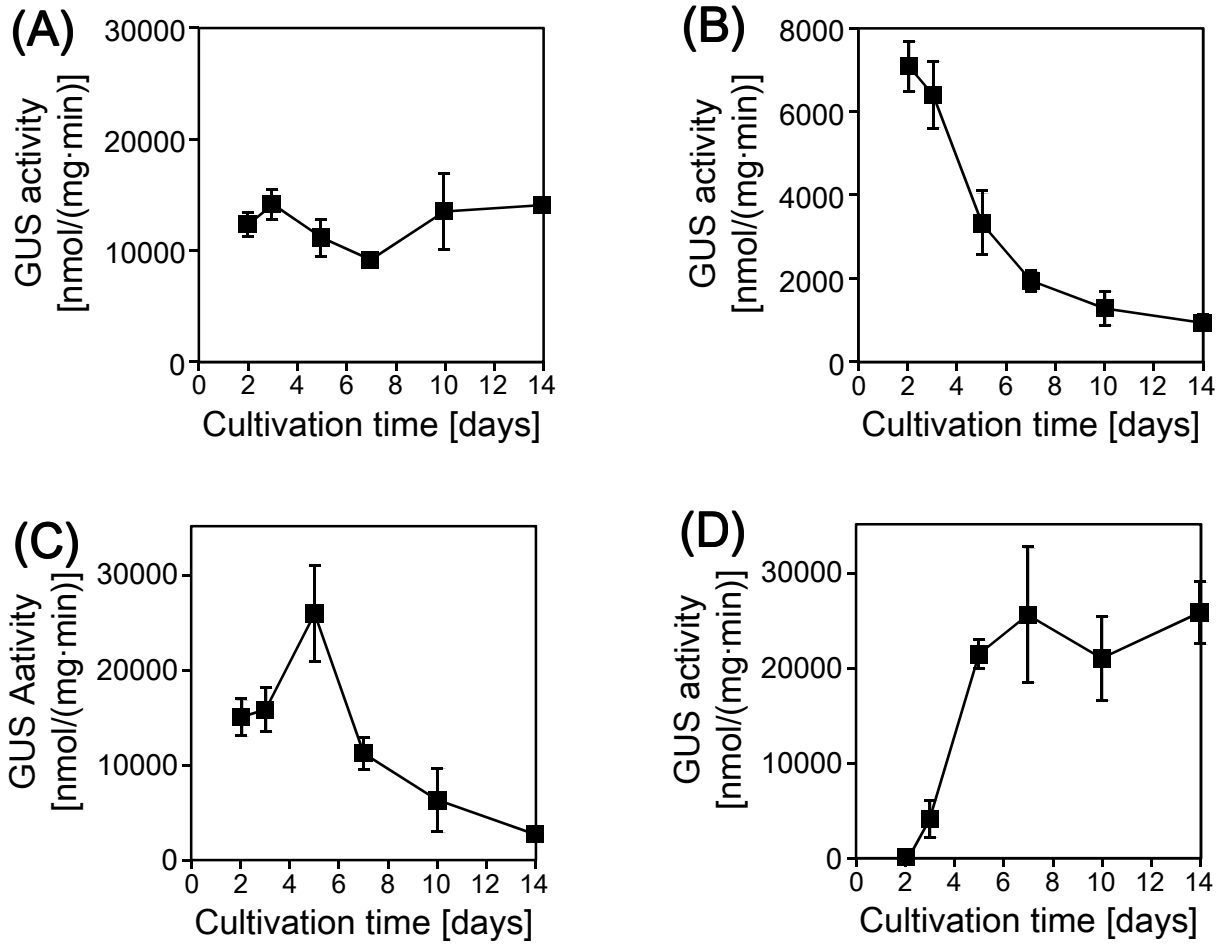
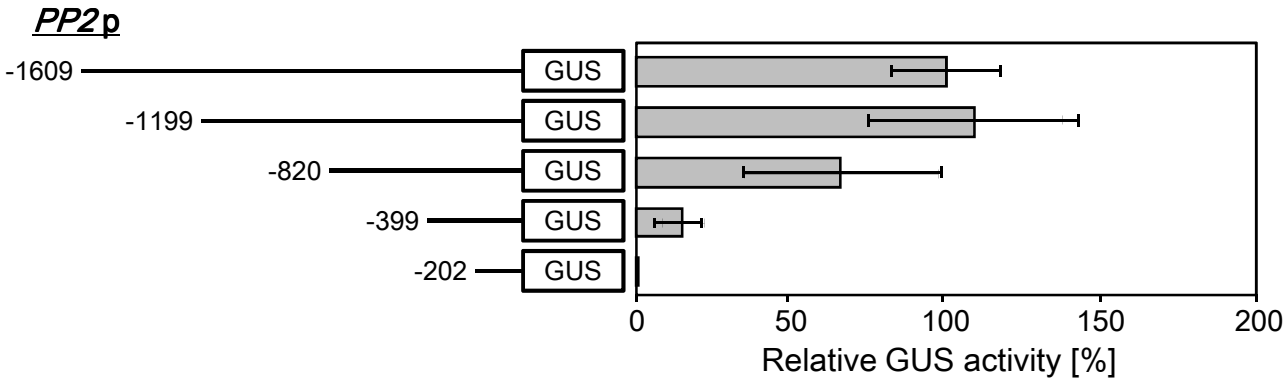
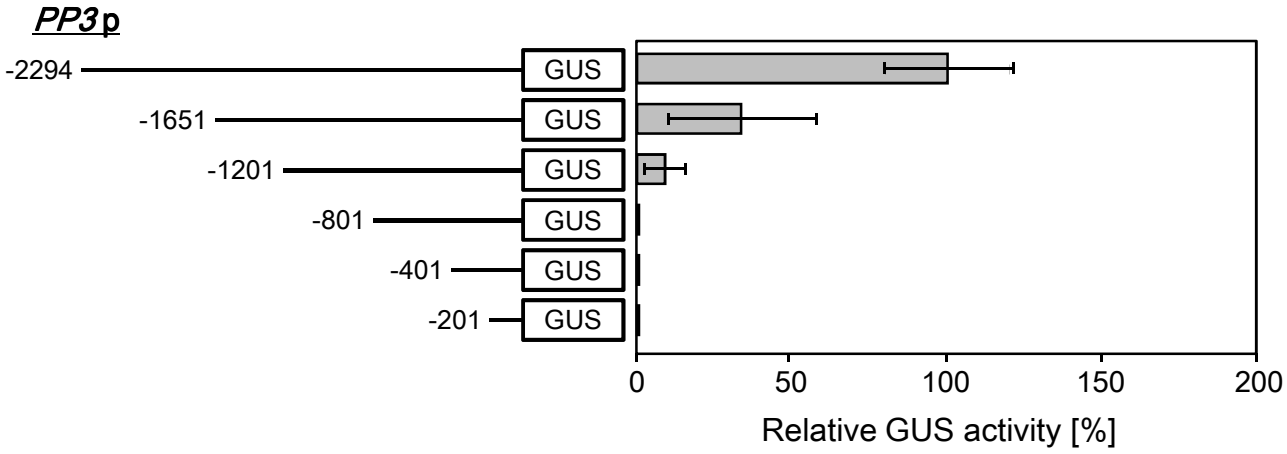
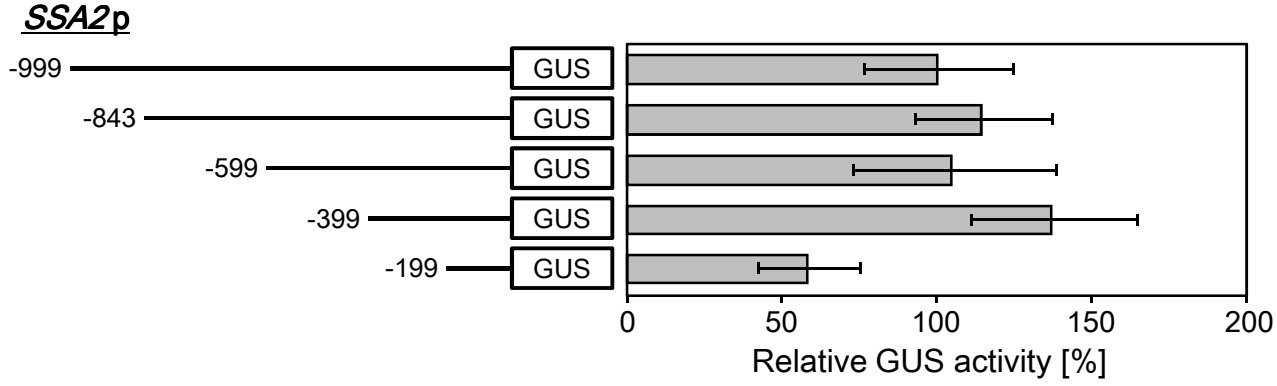
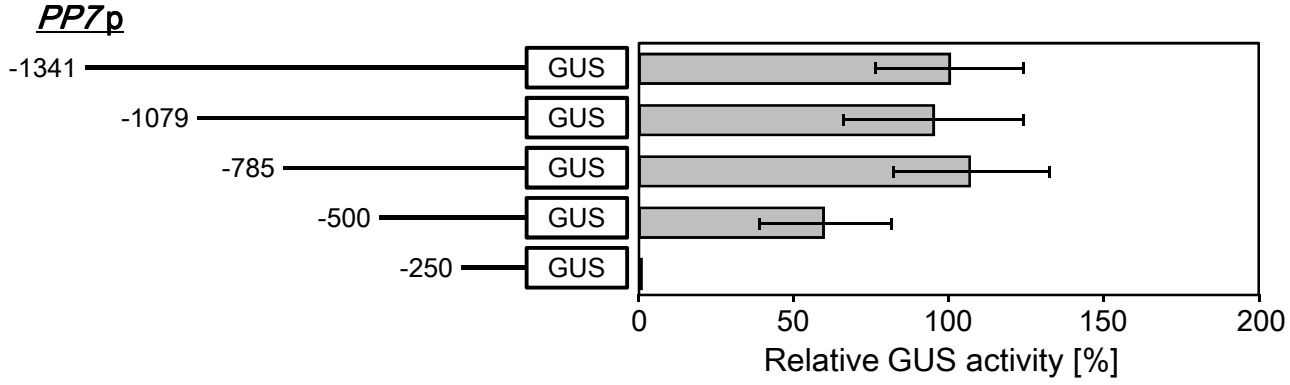


Fig. 4. Representative patterns of time-depend changes in GUS activity with different promoters. Results with (A) *HSC82*, (B) *CIT1*, (C) *PP6* and (D) *PP3* promoters are shown as representative. All transformants for each promoter construct were cultivated in GY medium for 2–14 d. GUS activity is expressed in nanomoles of *p*-nitrophenol produced per minute per milligram of protein. Plots represent the mean values with standard deviations of GUS activity in 3 individual transformant lines for each construct.


PP2p
-1609
-1199
-820
-399
-202
GUS
0
50
100
150
200
Relative GUS activity [%]


PP3p
-2294
-1651
-1201
-801
-401
-201
GUS
0
50
100
150
200
Relative GUS activity [%]


SSA2p
-999
-843
-599
-399
-199
GUS
0
50
100
150
200
Relative GUS activity [%]


PP7p
-1341
-1079
-785
-500
-250
GUS
0
50
100
150
200
Relative GUS activity [%]

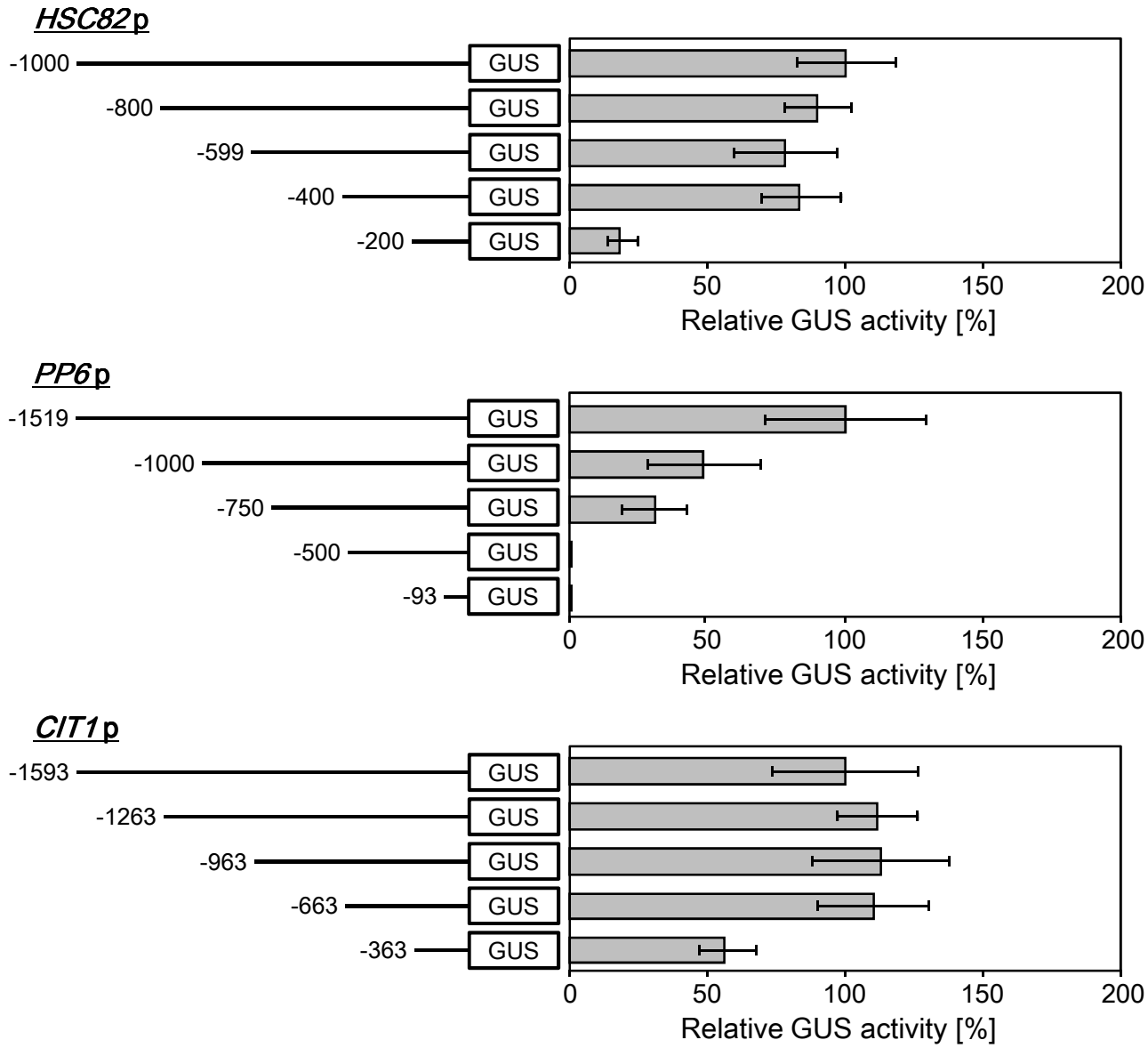


Fig. 5. 5′-deletion analysis of 7 different promoters. In the left column, constructs with different 5′ upstream deletions of individual promoters are shown. For each construct, the length of the fragment upstream from the transcription start site is shown on the left end. Putative PolII transcription factor binding sites are indicated by closed triangles. In the right column, GUS activity levels with the deleted constructs in *M. alpina* transformants are shown. All transformants were cultivated in GY liquid medium. Cultivation times were 5 d for the *SSA2*, *HSC82*, *PP7* and *PP6* promoters, 3 d for the *CIT1* promoter, and 14 d for the *PP2* and *PP3* promoters. The average GUS activity of each full-length construct is set at 100% and has been used to define the relative GUS activity of individual deletion constructs. Bars represent the mean values with standard deviations of GUS activity in 10 individual transformant lines for each construct.

Table S1. PCR primers for selected promoters

Promoter	Primer F sequence (5′- 3′)	Primer R sequence (5′- 3′)
PP1 p	AATCTCTAGA[a]GCGCAGTCGGAATGCC	AGTAACTAGTCGTGTTTTCTTTTGAAATGGG
PP2 p	AAGCTCTAGAGACTGTAAAGACGGAGGGG	AGTAACTAGTTGTGGATAGTGGGTAGTGG
PP3 p	AACGTCTAGACGTGTTATCTTGCGCTGC	TCATACTAGTGATGATTTAGAGGTGTTGG
SSA2 p	TTAGTCTAGAAAAGTGCTGCTTCGGAACC	AGATACTAGTGATGTAGATGTGAGTGTGAG
PP7 p	AATATCTAGATGACCGTGCGCTTTTTGAGAC	AGCAACTAGTCGTATATTTGTTGAAAGGTG
SSA22 p	AATATCTAGAGGGTGCAGGTCCGGTCC	AGCCACTAGTTCTACTCACCTTTTCCCTCAG
PP4 p	TGAGTCTAGAAGAGTGATTTTGTGGCTGTAC	CAATACTAGTGGCTGATGTATGTGTTGATG
PP8 p	ATGCTCTAGATATGGCGACCCATTCACG	AAGAACTAGTGGTTGAACAGAGTATGTTTGC
SAH1 p	AATCTCTAGACTGGCGAATACATGCGCAC	ATAGTCTAGAGGTGGATATGAAGGGTGG
PET9 p	ACCTTCTAGAAGACGAGAAGAGTTCATGATG	AATAACTAGTGATGAGTGTATGTGGAGAGTG
HSP104 p	AATATCTAGAGTTGAAGGTGCAGACACCGG	AATAACTAGTGGTGGGGCGTTATGTGG
HSC82 p	ATCATCTAGAGAGCTCAAGATGAAGGTGCTC	AATAACTAGTGGTGTGTGTGGTTTGCGGG
UBC5 p	AACTACTAGTGTATACAGGTCTTAGAGACC	ATTCACTAGTCGTGGGTGGAGAGAGTG
CDA1 p	AACTCTAGATGAAAATAGAAATGGGTGGATGG	ATTGACTAGTCGTAGGTTTCTTTGTGTGTG
RPP0 p	AATGTCTAGACACAGTGACAAGGGTGTTAAC	ATGCACTAGTGTTGATTATTGTTCGAGGG
PP5 p	AACGTCTAGATGTTTTTTGTGCAAATTACCTCG	AAGCACTAGTTTTGGATTGGGATTGCTTGAG
PP6 p	AAAGTCTAGACTGGCAATAGTTAGTGCACG	ATCAACTAGTGATGGAGGTTTGTTTGAGAAG
RPS16B p	AATGTCTAGACCTGCAGAAAGATGATCCAAAAG	AAGCACTAGTGATGAATAATGCCTATGATCAG
EFB1 p	TTAGACTAGTCGTAGTTGACTCTTTTATG	CAGTACTAGTGGTGGGTGCTTTGTCGATTTG
TDH1 p	AACCTCTAGAAGGAAATAAATTCTCCTCGGTG	AATAACTAGTGTTGAGTGGGTGTGTGTGG
CIT1 p	ATTTTCTAGACACCTCAAAAACGTGCCTTG	AATAACTAGTGGCGGATATGTGTATGGAG
TIF2 p	AAGTTCTAGAGTCGACCTATCATCATTTTTGGC	AGCGACTAGTGTTTTTTTTTGCTTTTTTTTTTTATG
CAT2 p	AATCACTAGTAAACGGTGGAGCATTCTCAC	TATCACTAGTGAAGGCGATGGGCAGGG
ELO1 p	AATGTCTAGACTTGCCCAGCATTACTCC	TCATACTAGTCTTTGAGGGGAGGAATTGC
IPP1 p	ACAATCTAGAGGCTGCGTTGCCGGGAG	ATAGACTAGTGGTGGTGGTGAAGAGTAG
OLE1 p	AGCATCTAGAGGGTTCTCACATTGAATTTG	AATAACTAGTCGCTGTGCGTCCTGCGTTG
PGK1 p	TGAATCTAGACACCGTCGCTATGTGAAG	TTGCTCTAGAGCAGAAACACACTGGCAG

[a] The underlined sequences show synthesized *Xba*I (TCTAGA) and *Spe*I (ACTAGT) sites.

Table S2. PCR primers used for deletion clones

Promoter	Length of deletion clone (bp)	Primer F sequence (5′- 3′)
PP2 p	1199	ATTTCTAGA[a]TGCATTTACAGGTGAATATTAC
	820	TTATCTAGACATAAAAGTGTCTGGAGCG
	399	TTATCTAGAACTAAGTGGTGTCTACTTTGG
	202	AATTCTAGAGGATACTCCATCCCCACCC
PP3 p	1651	AATATCTAGAGATCCTGGTCGAAAAAGACAG
	1201	AATGTCTAGATGAGTTTCTGTTTTTTCCTTTTTGC
	801	AATATCTAGATGAACAATTCATGCAGCTTCACG
	401	AATATCTAGACGTCTAAGCGTTTACGTGCC
	201	AATATCTAGACTCGTTTTGATGGAGTTCTC
SSA2 p	843	AGTATCTAGATGACGGCGTGTATATGTCAG
	599	AGGTTCTAGACCATTGTATCGATTTCTGAT
	399	AGTATCTAGAGCTATGCGAACGGTTCATTTTG
	199	AGGTTCTAGATTTTTTCTCTCTGGTGTGAACG
PP7 p	1079	AGCATCTAGAAAAACTATTCAATAATGGGCG
	785	ATTTCTAGAATGGCGAGACGCAGGGGGTAG
	500	AATATCTAGAGAGTGGGCACTGAACTAAAAAG
	250	AATATCTAGAGACACTGCATGACGCGAAATC
HSC82 p	800	AATTCTAGATTTTACTACCGCATTCCCTTTTC
	599	ACGTCTAGACCTTTTCAGTAAACAATTTC
	400	ATTTCTAGACACAAAGAAGAAGGGTGTGTC
	200	ACGTCTAGAACTGTTTTCTTGAAACTTC
PP6 p	1000	AATTCTAGACAGTTACCGTGCGCCCACTG
	750	AATTCTAGACTTTCACAAATAGGCATCCTATC
	500	AATTCTAGAGGCTTTTTCGTTTATTGGATTG
	93	ACGTCTAGATATCCAATTCTCACCACTTC
CIT1 p	1263	AAGTCTAGATGTCAATCATCTTTGCTGCTG
	963	TGCGTCTAGAATTATAATTATAATGAGGAAGTG
	663	TTATCTAGAGGCGAGTGGCGGACTGC
	363	TTGTCTAGACAATTGGCAAGGCTGGGTTG

[a] The underlined sequences show synthesized *Xba*I (TCTAGA) site.