Improvement of ligninolytic properties in the hyper lignin-degrading fungus Phanerochaete sordida YK-624 using a novel gene promoter

Research Letter

Improvement of ligninolytic properties in the hyper lignin-degrading fungus *Phanerochaete sordida* YK-624 using a novel gene promoter

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2 Running title: Improvement of ligninolytic properties in white-rot fungus

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Abstract

We identified a highly expressed protein (BUNA2) by two-dimensional gel electrophoresis from the hyper lignin-degrading fungus *Phanerochaete sordida* YK-624 under wood-rotting conditions. Partial amino acid sequences of BUNA2 were determined by LC-MS/MS analysis, and BUNA2 gene (*bee2*) and promoter region were PCR cloned and sequenced. The *bee2* promoter was used to drive expression of the manganese peroxidase gene (*mnp4*) in *P. sordida* YK-624. Eighteen *mnp4*-expressing clones were obtained, with most showing higher ligninolytic activity and selectively than wild-type YK-624. Examination of the ligninolytic properties of the most effective lignin-degrading transformant, BM-65, cultured on wood meal revealed this strain exhibited higher lignin degradation and MnP activities than those of wild type. Transcriptional analysis confirmed the elevated expression of recombinant *mnp4* in the transformant. These results indicate that use of the *bee2* promoter to drive expression of ligninolytic enzymes may be an effective approach for improving the lignin-degrading properties of white-rot fungi.

Introduction

Ethanol production from woody biomass has recently received increasing attention due to the sustainable availability of large quantities of raw materials and avoidance of competition for the use of food products (Festal, 2008). The biological conversion of woody biomass to ethanol involves several steps, including the pretreatment of raw materials, enzymatic hydrolysis of resulting cellulose fractions, glucose fermentation, and ethanol recovery. The pretreatment step is essential to improve the accessibility of cellulose to hydrolytic enzymes and has been studied intensively (Hendriks and Zeeman, 2009). Particularly, lignin, which is a heterogeneous, random, phenylpropanoid polymer, has been identified as a major deterrent to enzymatic hydrolysis of lignocellulosic biomass because of its close association with cellulose microfibrils (Berlin *et al*., 2006; Ximenes *et al*., 2011). As it constitutes 20%-30% of woody plant cell walls, the removal of lignin is necessary for the efficient production of ethanol from woody biomass.

Many woody biomass pretreatment methods, including physical, chemical, and biological approaches, have been studied and remain in development. It is difficult to evaluate and compare pretreatment technologies because they involve upstream and

downstream processing costs, capital investment, chemical recycling, and waste treatment systems (Jeoh *et al*., 2007). As white-rot basidiomycetous fungi are the only known microorganisms that are capable of degrading lignin extensively to CO_2 and H_2O (Kirk and Farrell, 1987), the abilities of these fungi are attracting interest as a pretreatment strategy for lignin elimination.

To degrade lignin, white-rot fungi produce multiple extracellular ligninolytic enzymes, which are separated into four major families: laccase, manganese peroxidase (MnP), lignin peroxidase (LiP) (Gold and Alic, 1993), and versatile peroxidase (Ruiz-Dueñas *et al*., 2001; Kamitsuji *et al*., 2005). The white-rot fungus *Phanerochaete sordida* YK-624, which was isolated from rotted wood, exhibits greater ligninolytic activity and selectivity among beech woods than either *Phanerochaete chrysosporium* or *Trametes versicolor* (Hirai *et al*., 1994). In a previous study, we demonstrated that *P. sordida* YK-624 produces MnP (Hirai *et al*., 1994 and 1995) and LiP (Sugiura *et al*., 2003; Machii *et al*., 2004; Hirai *et al*., 2005) as ligninolytic enzymes.

Recently, gene transformation systems for several species of white-rot fungi have been developed for the over-production of ligninolytic enzymes and facilitating structure-function studies of these enzymes by site-directed mutagenesis (Mayfield *et al*., 1994; Tsukamoto *et al*., 2003; Tsukihara *et al*., 2006). We previously constructed a

gene transformation system for *P. sordida* YK-624 using the glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) promoter for the heterologous expression of enhanced green fluorescent protein (EGFP) (Yamagishi *et al*., 2007) and the homologous expression of recombinant LiP (Sugiura *et al*., 2009). Notably, the ligninolytic activity and selectivity of the transformant expressing LiP were markedly higher than those of wild-type (Sugiura *et al*., 2010). However, explorations of more effective expression promoters and investigations of proteins involved in lignin degradation are essential to breedings of superior lignin-degrading fungi.

In the present study, we attempted to isolate the promoter region of a protein that is highly expressed by *P. sordida* YK-624 under wood-rotting conditions for the over-production of ligninolytic enzymes using this promoter in woody biomass cultivation. Moreover, the ligninolytic properties of a transformant that over-produces MnP under wood-rotting conditions were examined in detail.

Materials and Methods

Strains

P. sordida YK-624 (ATCC 90872), uracil auxotrophic strain UV-64 (Yamagishi *et al*., 2007), recombinant YK-LiP2-overexpression transformant A-11 (Sugiura *et al*., 2009), and *P. chrysosporium* ME-446 (ATCC 34541) were used in this study.

Protein extraction from P. sordida YK-624 cultivated on beech wood meal

A suspension consisting of 1 g ethanol-treated beech wood meal (60-80 mesh) and 2.5 ml distilled water in a 100-ml Erlenmeyer flask was inoculated with *P. sordida* YK-624 and then incubated at 30 °C for 10 days. Proteins were extracted from four fungal-inoculated wood meal suspensions by adding 100 ml extraction buffer (50 mM sodium phosphate, 0.5 mM phenylmethylsulfonyl fluoride, and 0.05% Tween 80) and stirring for 2 h at 4 °C. Soluble proteins were separated by filtering the suspension through a 0.2-μm membrane filter (Advantec). For the removal of phenolic compounds, 1 g acid-treated polyvinyl polypyrrolidone (Charmont *et al*., 2005) was added to the solution over a 2-h period with constant stirring at 4 °C, and residue was removed by filtering. Proteins precipitated between 30% and 80% saturation of ammonium sulfate

were obtained by centrifugation of the solution at 15000 × *g* for 30 min at 4 °C. The resulting pellet was dissolved in 50 ml H_2O, and trace ammonium sulfate and contaminants were then removed by repeated (two times) concentration (Advantec, 10-kDa cut off) and resuspension of the proteins in 10 ml H_2O. Protein content was measured using a Bio-Rad protein assay kit.

Electrophoresis, In-gel digestion and LC/MS/MS analysis

The sample was precipitated and dissolved in Reagent3 (Bio-Rad). Details were described in Appendix S1. The solution was used to rehydrate an IPG ReadyStrip (7 cm, pH 3-10; Bio-Rad). The first-dimensional isoelectric focusing (IEF) was focused in three steps at 150 V (15 min), 150-4000 V (2 h), and 4000 V (8 h) using a Protean IEF cell (Bio-Rad).

Equilibration and SDS-PAGE were performed according to the manufacturers' instructions with 10% SDS-PAGE gel on a Mini-PROTEAN Tetra cell (Bio-Rad) at 150V. The gel was stained with SYPRO Ruby Protein Gel Stain (Molecular Probes) following the manufacturer's guidelines. Relative fluorescence intensities were calculated using Image J software (http://rsbweb.nih.gov/ij/). In-gel digestion and LC/MS/MS analysis of that were performed as previously described (Ogata *et al*., 2010)

with some modifications (Appendix S1).

Cloning procedure for the full-length gene encoding BUNA2 and 5’ flanking region

Total RNAs were extracted from inoculated wood meal suspensions using Plant RNA Isolation Reagent (Invitrogen) and purified with a RNeasy Plant Mini kit (Qiagen) according to the manufacturers’ instructions. A cDNA encoding BUNA2 was cloned by a series of PCR procedures using the primers listed in Table S1. The 3’-coding region of the gene was cloned by 3’-rapid amplification of cDNA ends (RACE) using a 3’-Full RACE core set (TaKaRa Bio) and primer BUNA2dF and sequenced. The 5’-coding region was cloned by 5’-RACE using a 5’-Full RACE core set (TaKaRa Bio) and 5’-phosphorylated primer 5phosBUNA2R and two nested primer sets, corresponding to 3’-RACE PCR fragments BUNA2F1-BUNA2R1 and BUNA2F2-BUNA2R2.

Genomic DNA was isolated from *P. sordida* YK-624 mycelium using ISOPLANT II (Nippon Gene). TAIL-PCR was performed using the degenerate primers TAIL1 - 6, as described previously (Yamagishi *et al*., 2007), to obtain the 5’ flanking region of *bee2*. Nested primers BUNA2R1, R2, and R3 were used as gene-specific primers. Inverse PCR was performed to further upstream of the 5’ flanking region using the primer sets bee2proF1-bee2proR1 and bee2proF2-bee2proR2, and the restriction

enzyme SacII (New England Biolabs), as previously described (Ochman *et al*., 1988). The full-length 5' flanking region of *bee2* (1584 kb) was amplified using primer sets bee2proF1-bee2proR1.

Construction of MnP gene expression vector, co-transformation of UV-64 and screening of regenerated clones

The procedure for constructing the MnP gene (*mnp4*) expression plasmid, pBUNA2pro-mnp4, was shown in Figure S1 and details were described in Appendix S1.

UV-64 protoplasts were prepared and then transformed with pPsURA5 and pBUNA2pro-mnp4 using standard techniques. The co-transformed clones were selected by PCR, as described previously (Sugiura *et al*., 2009), with the following modifications: primers bee2proF4 and mnp4R3 were designed to amplify the *mnp4* gene fused with the *bee2* promoter.

Screening based on ligninolytic properties

P. chrysosporium ME-446, *P. sordida* YK-624, and the transformants A-11 and

24 were cultured in suspensions consisting of 0.5 g extractive-free beech wood meal (60-80 mesh) and 1.25 ml distilled water in 50-ml Erlenmeyer flasks, which were then incubated at 30 °C for 28 days. After incubation, weight loss, Klason lignin content, and acid-soluble lignin content of the fungal-treated wood meal suspensions were determined, as previously described (Hirai *et al*., 1994). The selection factor (SF), which is an indicator of ligninolytic selectively, was calculated as follows: SF = lignin loss / holocellulose loss. Holocellulose loss was calculated as follows: total weight loss - lignin loss.

Detailed determination of ligninolytic properties

P. chrysosporium ME-446, *P. sordida* YK-624 and BM-65 were cultured in wood meal suspensions, as described above, and were incubated at 30 °C for 7, 14, 21, and 28 days. After incubation, weight loss, Klason lignin content, and acid-soluble lignin content of the fungal-treated wood meals were determined, as described above.

Enzyme extraction and assay

P. sordida YK-624 and BM-65 were cultured in wood meal suspensions, as described above, and were incubated at 30 °C for 4, 8, 12, 16, 20, 24, and 28 days.

Following the culture period, the method described by Hirai *et al*. (1994) was modified for enzyme extraction. Briefly, fungal-treated wood meal was homogenized with 25 ml of 50 mM malonate buffer (pH 4.0) containing 0.05% Tween 20 (Wako) using a Polytron PT1200 homogenizer for a total of 5 min (20 s blending with 10 min intervals) at 4 °C.

Modified methods described by Périé and Gold (1992) and Wariishi *et al*. (1994) were used for the determination of MnP and LiP activities, respectively and details were described in Appendix S1.

Transcriptional analysis of BM-65

P. sordida YK-624 and BM-65 were cultured in wood meal suspensions, as described above, were incubated at 30 °C for 4, 8, 12, 16, 20, 24, and 28 days. Fungal-treated wood meals were stored at -80 °C. The purification of total RNA from the two fungal cultures was performed as described above. The concentration and purity of total RNA was estimated by measuring the absorbance at 260 and 280 nm. Two hundred nanograms of total RNA was reverse transcribed using a Takara Prime Script RT-PCR kit (TaKaRa Bio). The synthesized cDNA was amplified by PCR using a LightCycler System (Roche Applied Science) with primer pairs targeting native *mnp4*

(mnp4F2-mnp4R4) and recombinant *mnp4* (mnp4F2-gpdR1), and *gpd* (gpdF1-gpdR2), which was used as an endogenous reference gene. Details of primers design and the LightCycler reaction were described in Appendix S1.

Nucleotide sequence accession numbers

The nucleotide sequences of the gene *mnp4*, full-length cDNA of *bee2* and 5' flanking region of *bee2* derived from *P. sordida* YK-624 have been deposited in the DDBJ database (http://www.ddbj.nig.ac.jp/) under accession nos. AB585997, AB638492 and AB638493, respectively.

Result and Discussion

Identification of highly expressed proteins from *P. sordida* YK-624 in wood meal

When *P. sordida* YK-624 was cultured under wood-rotting conditions, large amounts of proteins were produced, as determined by 2-DE. In the protein sample extracted from the wood-meal culture incubated for 10 days, three strong and sharp spots were observed in the 2-DE gel (Fig. 1). The three spots exhibited high relative fluorescence intensity (1, 0.72; 2, 0.63; and 3, 0.63) compared with the 50-kDa band of the molecular marker (0.3 μg). The protein spots 1, 2, and 3 were named BUNA1, BUNA2, and BUNA3, respectively.

In the LC-MS/MS analysis for BUNA2, five fragments were identified by an MS/MS ion search on the Mascot on-line server (Table S2). However, the proteins identified based on these peptide fragments were not consistent with one another. Thus, *de novo* sequencing was performed using Peaks Studio software and the amino acid sequences of 14 fragments were predicted for BUNA2 (Table S3).

Isolation of the BUNA2 gene from *P. sordida* YK-624

The results of the LC-MS/MS analysis indicated that BUNA2 was a protein of

unknown function. Cloning of the gene encoding this protein was needed to acquire the promoter region regulating BUNA2 expression. The degenerate primer BUNA2dF, designed based on the fragment NPVDWK was used to perform 3'-RACE PCR. Upon sequencing of the PCR product, nine fragments identified by LC-MS/MS analysis were included in the deduced amino acid sequence of that. We concluded that the obtained cDNA encoded the BUNA2 gene, which was designated *bee2*. The full-length cDNA and 5' flanking region of the genomic DNA of *bee2* were cloned by a combination of 5'-RACE, TAIL, and inverse PCR procedures. Sequencing of the obtained PCR products revealed the full-length cDNA of *bee2* is 1166 bp and GC-rich (68%). In addition, 13 fragments identified in LC-MS/MS analysis were corresponded.

The deduced amino acid sequence of BUNA2 was compared with the genome database of *P. chrysosporium*. BUNA2 showed the highest identity with fgenesh1_pg.C_scaffold_4000081 (73%, Fig. 2). Based on the annotation results of the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml), BUNA2 was classified as a possible enoyl reductase of the medium-chain dehydrogenase/reductase (MDR) family. The MDR superfamily with ~350-residue subunits contains the classical liver alcohol dehydrogenase (ADH), quinone reductase, and leukotriene B4 dehydrogenase, in addition to numerous other forms. (Persson *et al.*,

2008).

In 2004, a nearly complete annotation of the *P. chrysosporium* genome was made publicly available by the US Department of Energy (DOE) and the Joint Genome Institute (Martinez *et al*., 2004) (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html). Using this database, a number of proteomic and transcriptomic analyses of *P. chrysosporium* cultured under various conditions have been performed. In the case of proteomic analysis, differential displays were performed in liquid medium supplemented with vanillin (Shimizu *et al*., 2005) or benzoate (Matsuzaki *et al*., 2007), and proteome mappings were performed in soft wood meals or cellulose as a carbon source (Abbas *et al*., 2005; Wymelenberg *et al*., 2005; Sato *et al*., 2007; Ravalason *et al*., 2008). In the transcriptomic analyses, mRNA was extracted in liquid medium containing hardwood and identification of transcripts was accomplished by pyrosequencing (Sato *et al*., 2009). Despite these numerous analyses, the expression or transcription of fgenesh1_pg.C_scaffold_4000081 was not observed. Taken together with our present results, these findings suggest that the high-level expression of BUNA2 is unique to *P. sordida* YK-624, and furthermore, it is possible that BUNA2 is one of the key proteins required for the high ligninolytic activity of *P. sordida* YK-624.

Construction of a *mnp4*-overexpression transformant and screening of ligninolytic properties

A plasmid for the overexpression of *mnp4* was constructed from p*PsGPD-EGFP* (Yamagishi *et al*., 2007) by inserting genomic DNA of *mnp4* between the *bee2* promoter and *gpd* terminator (Fig. 3a). The expression plasmid, pBUNA2pro-mnp4, was introduced into UV-64 using p*PsURA5* as the marker plasmid. The presence of the *bee2* promoter-*mnp4* fusion gene in each uracil prototrophic clone was confirmed by PCR using genomic DNA as the template (Fig. 3b).

Eighteen regenerated clone was cultured on beech wood meal and ligninolytic activity was determined after 28 days based on the percentage of lignin degradation (Fig. 3c). The results indicated that most of the transformants displayed higher ligninolytic activity and selectively than the wild-type and A-11 strains. The most effective lignin-degrading transformant was BM-65, and was therefore used for subsequent analyses.

Ligninolytic properties and transcriptional analysis of BM-65

The effect of MnP over-expression was investigated by determining the ligninolytic properties of strain BM-65 cultured on beech wood meal. Strain BM-65

showed 1.22-fold higher ligninolytic activity after 4 weeks (Fig. 4a). The SF values of BM-65, the wild-type strain, and *P. chrysosporium* are shown in Table 1. BM-65 showed higher SF values than the wild-type strain during the entire incubation period. Taken together, these results suggest that the ligninolytic properties of BM-65 were improved by overexpressing MnP under control of the *bee2* promoter.

To confirm whether the improvement of the ligninolytic properties resulted from an increase of MnP production, MnP and LiP activities in beech wood meals inoculated with BM-65 and the wild-type strain were determined. The LiP activity of BM-65 was similar to that of wild type, and no drastic fluctuations were observed (Fig. 4b). In contrast, although similar MnP activities for each strain were detected on days 4 and 8, significantly higher activity was detected at days 12 and 16 in BM-65 (Fig. 4c) and the fold increase were 9.0 nkat and 5.2 nkat, respectively. Katagiri *et al*. (1994) reported that a linear relationship between pulp brightness increase and cumulative MnP activity was found in a solid fermentation system using hardwood unbleached kraft pulp. The results of the present study are consistent with that report; thus, our results suggest that the improvement of ligninolytic activity in BM-65 was attributed to increased MnP production, particularly in the intermediate stages of the culture. Furthermore, the elevated production of MnP resulting from gene transfection appears to be effective for

the enhancement of ligninolytic activity.

Transcriptional analysis was performed by real-time PCR to confirm whether the increment of MnP production was caused by the *bee2* promoter-regulated expression. *gpd*, the only housekeeping gene cloned from this strain was used as an internal control. For native *mnp4*, the transcription level at day 4 was the highest in each strain and markedly decreased from day 8 (Fig. 5a). Janse *et al*. (1998) reported that transcription of all MnP isozymes at two weeks were higher than those at eight weeks in *P. chrysosporium* grown on hardwood meal. This observation was consistent with the results of our present transcriptional analysis of native *mnp4* in *P. sordida* YK-624. In contrast to native *mnp4*, we observed high levels of recombinant *mnp4* transcription from days 4 to 16 days in BM-65 (Fig. 5b). These results suggest that the transcription of recombinant *mnp4* is involved in the increase of MnP production in beech wood meal. Thus, the *bee2* promoter is more useful than the GPD promoter under wood-rotting conditions.

To conclude, we identified a protein BUNA2, which was highly produced by *P. sordida* YK-624 under wood-rotting conditions. The promoter region of the BUNA2 gene, designated *bee2*, was successfully cloned and demonstrated to be a useful regulator for the high expression of genes under conditions suitable for lignin

degradation. In addition, we found that the over-expression of *mnp4* under control of the *bee2* promoter is effective for improving the ligninolytic properties in this fungus. Thus, the molecular breeding of superior lignin-degrading fungi for the pretreatment of woody biomass in the production of bioethanol is possible by the high-expression of multiple ligninolytic enzyme genes driven by the *bee2* promoter.

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Figure titles

Fig. 1. Two-dimensional gel electrophoresis of extracted proteins from beech wood meal cultures inoculated with *P. sordida* YK-624. A standard protein size marker was run in the left lane of the gel.

Fig. 2. Amino acid sequence alignments of BUNA2 and fgenesh1_pg.C_scaffold_4000081. Identical and positive residues are shown in black and gray boxes, respectively. Straight and dotted lines above BUNA2 indicate sequences that are completely and partially consistent, respectively, with the fragments determined by *de novo* sequencing.

Fig. 3. Co-transformation of UV-64 with the pBUNA2pro-mnp4 expression plasmid. (a) Restriction map of the LiPH8 expression plasmid pBUNA2pro-mnp4. (b) Detection of *mnp4* linked with the *bee2* promoter from 18 regenerated clones co-transformed with pPsURA5 and pBUNA2pro-mnp4 by PCR. A 100-bp ladder size marker was loaded in lane M. The clone numbers are indicated above the gel lanes. (b) Lignin degradation rate and selection factor of transformants cultured on beech wood meals for 28 days.

White and black bars indicate lignin degradation rate and selection factor, respectively. P.c., *P. chrysosporium* ME-446; YK-624, *P. sordida* YK-624.

Fig. 4. Ligninolytic properties of strain BM-65 (square), *P. sordida* YK-624 (rhombus), and *P. chrysosporium* ME-446 (triangle). (a) Lignin degradation of beech wood meal by each strain. (b) LiP and (c) MnP activities detected in fungal-treated beech wood meals. Values are means ± standard deviations of two duplicates.

Fig. 5. Relative transcription levels of (a) native *mnp4* and (b) recombinant *mnp4* in fungal-treated beech wood meals. White and black bars indicate *P. sordida* YK-624 and BM-65, respectively. Gene expression was determined by real-time RT-PCR and normalized to *gpd* expression.

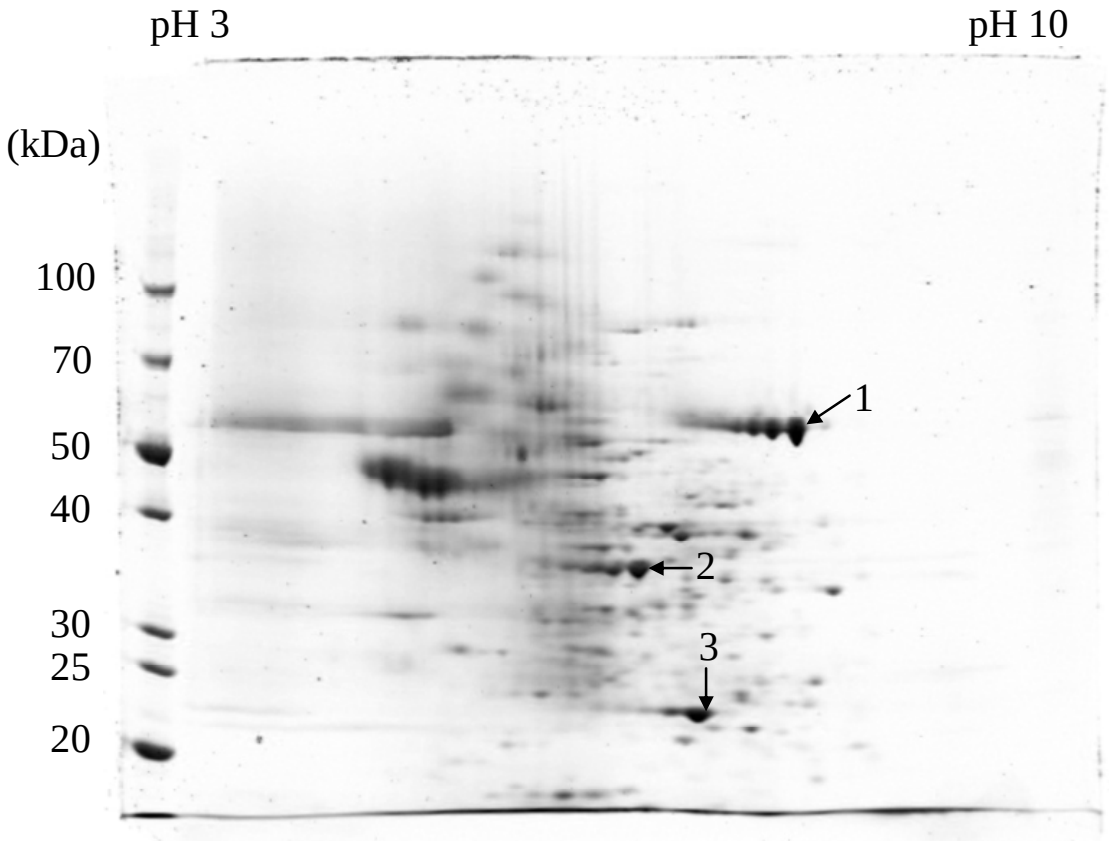
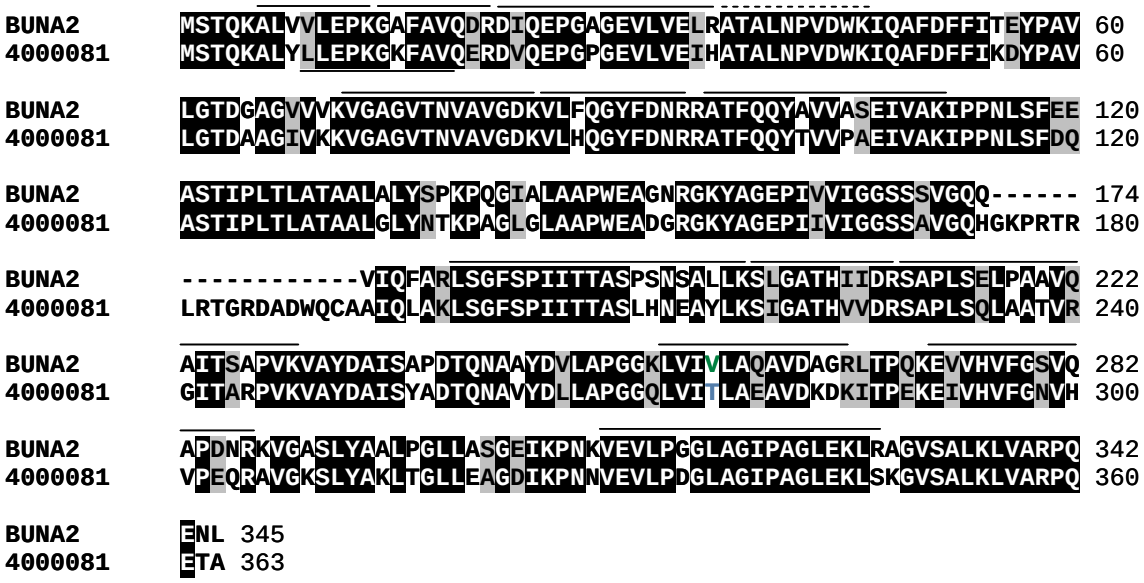


Fig. 1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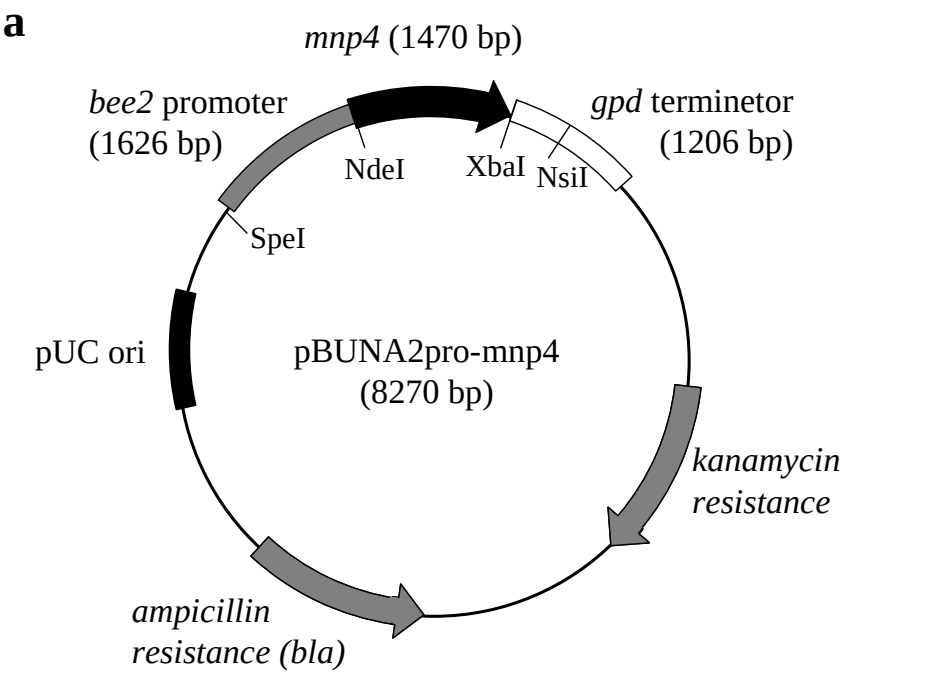
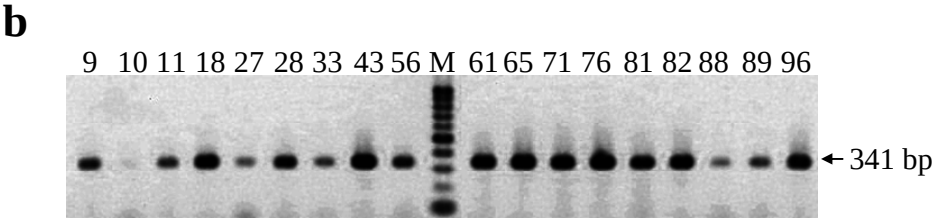
4000081 MSTQKALYLLEPKGKFAVQERDVQEPGPGEVLVEIHATALNPVDWKIQAFDFFIKDYPAV 60
BUNA2 LGTDGAGVVVKVGAGVTNVAVGDKVLFQGYFDNRRATFQQYAVVASEIVAKIPPNLSFEE 120
4000081 LGTDAAGIVKKVGAGVTNVAVGDKVLHQGYFDNRRATFQQYTVVPAEIVAKIPPNLSFDQ 120
BUNA2 ASTIPLTLATAALALYSPKPQQGIALAAPWEAGNRGKYAGEPIVVIGGSSSVGQQ------ 174
4000081 ASTIPLTLATAALGLYNTKPAGLGLAAPWEADGRGKYAGEPIIVIGGSSAVGQHGKPRTR 180
BUNA2 ------------VIQFARLSGFSPIITTASPSNSALLKSLGATHIIDRSAPLSELPAAVQ 222
4000081 LRTGRDADWQCAAIQLAKLSGFSPIITTASLHNEAYLKSIGATHVVDRSAPLSQLAATVR 240
BUNA2 AITSAPVKVAYDAISAPDTQNAAYDVLAPGGKLVIVLAQAVDAGRLTPQKEVVHVFGSVQ 282
4000081 GITARPVKVAYDAISYADTQNAVYDLLAPGGQLVITLAEAVDKDKITPEKEIVHVFGNVH 300
BUNA2 APDNRKVGASLYAALPGLLASGEIKPNKVEVLPGGLAGIPAGLEKLRAGVSALKLVARPQ 342
4000081 VPEQRAVGKSLYAKLTGLLEAGDIKPNNVEVLPDGLAGIPAGLEKLSKGVSALKLVARPQ 360
BUNA2 ENL 345
4000081 ETA 363

Fig. 2


a
mnp4 (1470 bp)
bee2 promoter
(1626 bp)
gpd terminetor
(1206 bp)
NdeI
XbaI
NsiI
SpeI
pUC ori
pBUNA2pro-mnp4
(8270 bp)
kanamycin
resistance
ampicillin
resistance (bla)


b
9 10 11 18 27 28 33 43 56 M 61 65 71 76 81 82 88 89 96
341 bp

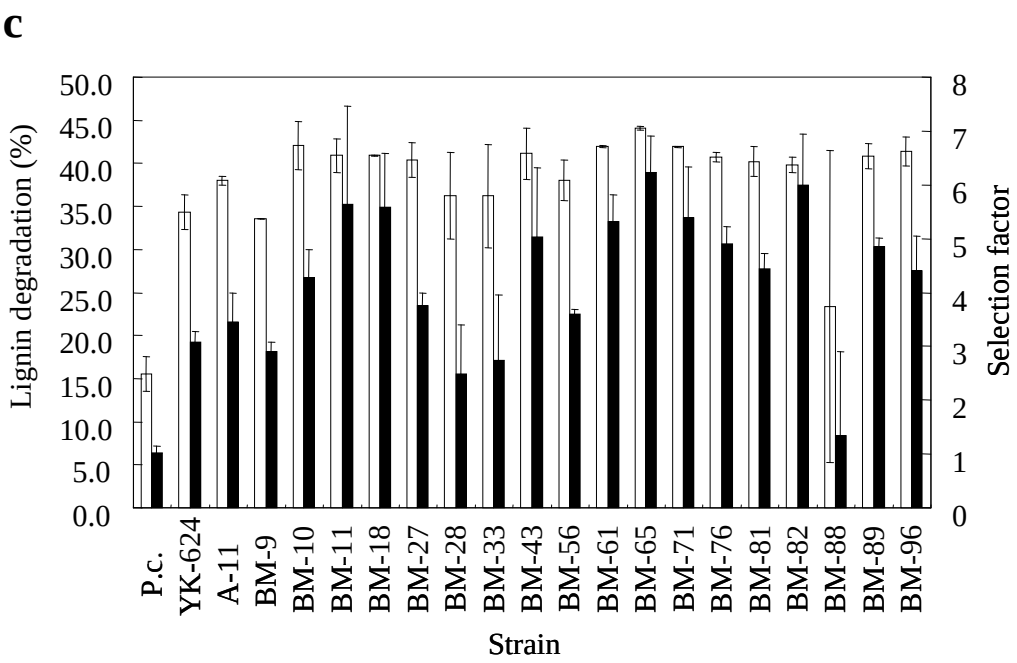

c
Lignin degradation (%)
Selection factor
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
8
7
6
5
4
3
2
1
0
P.c.
YK-624
A-11
BM-9
BM-10
BM-11
BM-18
BM-27
BM-28
BM-33
BM-43
BM-56
BM-61
BM-65
BM-71
BM-76
BM-81
BM-82
BM-88
BM-89
BM-96
Strain

Fig. 3

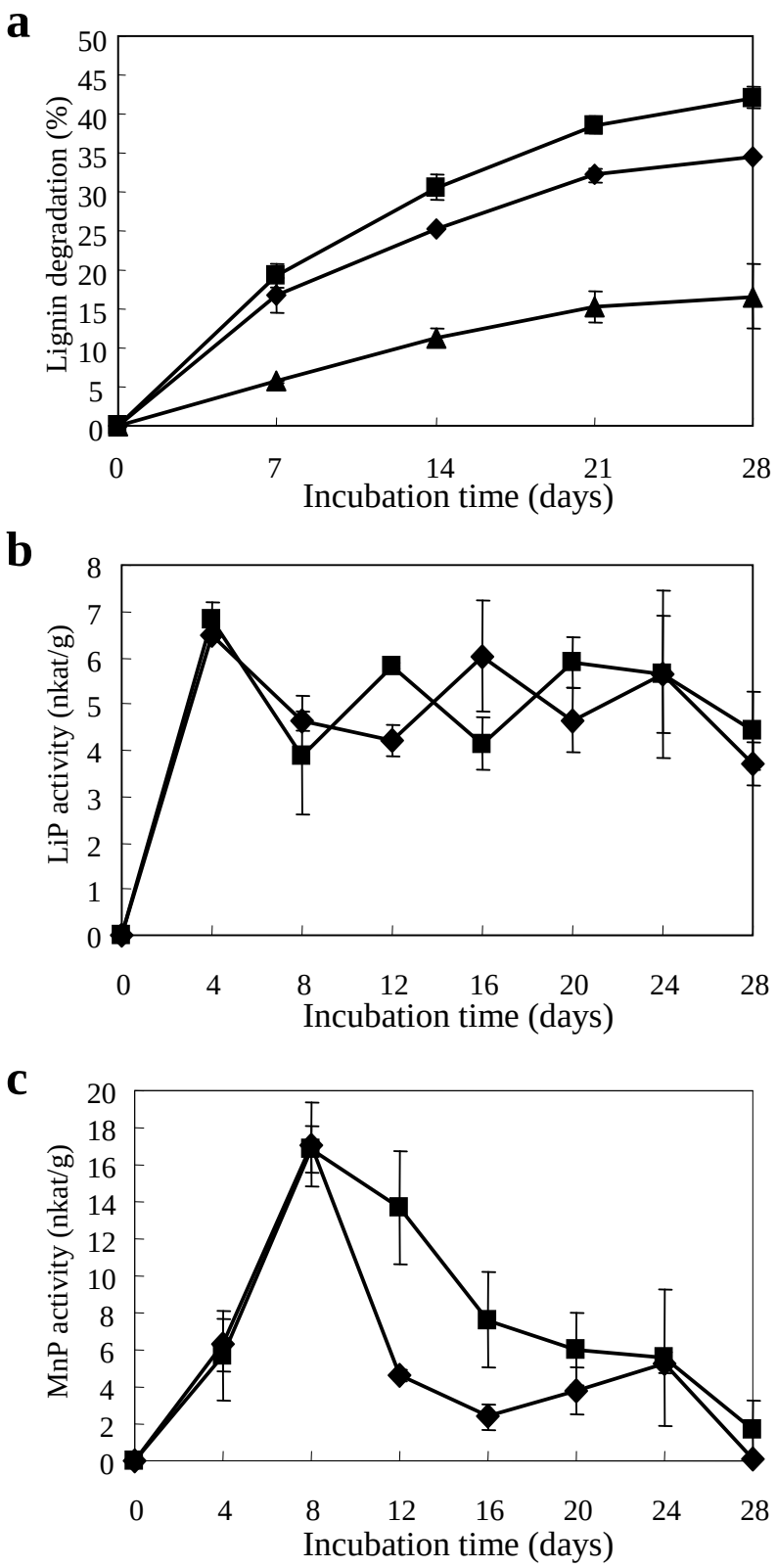

a
Lignin degradation (%)
0
5
10
15
20
25
30
35
40
45
50
0
7
14
21
28
Incubation time (days)
b
LiP activity (nkat/g)
0
1
2
3
4
5
6
7
8
0
4
8
12
16
20
24
28
Incubation time (days)
c
MnP activity (nkat/g)
0
2
4
6
8
10
12
14
16
18
20
0
4
8
12
16
20
24
28
Incubation time (days)

Fig. 4

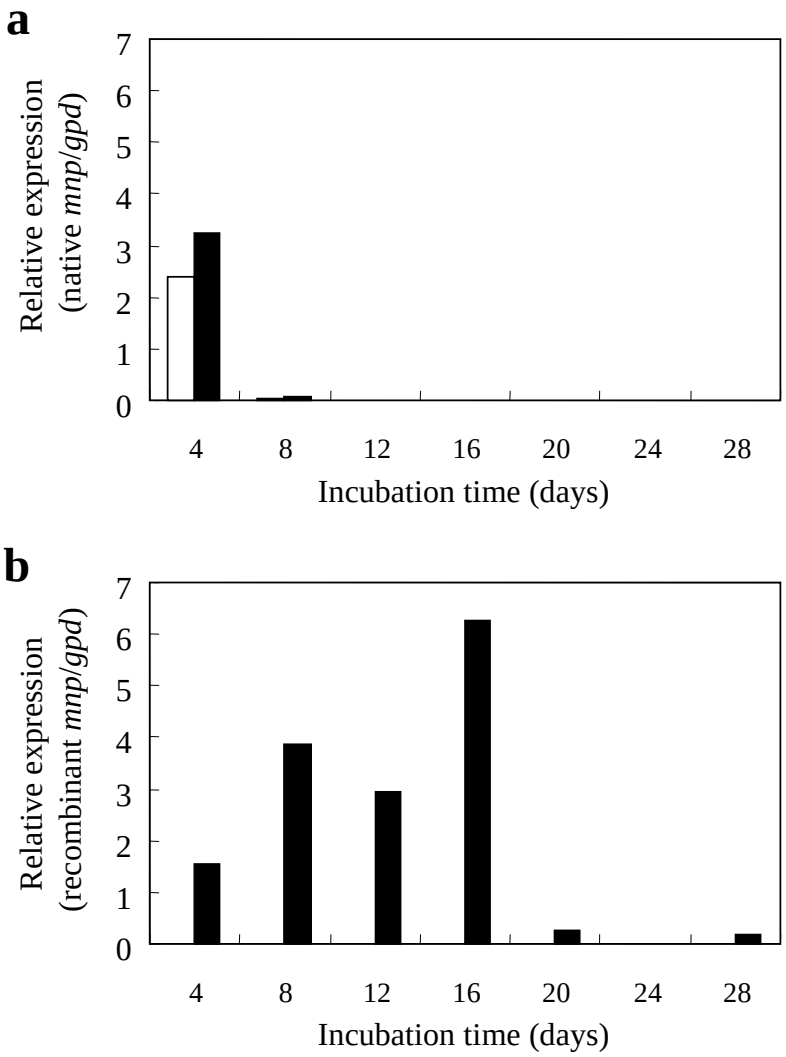

a
Relative expression
(native *mnp/gpd*)
0
1
2
3
4
5
6
7
4
8
12
16
20
24
28
Incubation time (days)
b
Relative expression
(recombinant *mnp/gpd*)
0
1
2
3
4
5
6
7
4
8
12
16
20
24
28
Incubation time (days)

Fig. 5

Table 1. Selection factor of wild-type *P. sordida* YK-624 (WT), BM-65, and *P. chrysosporium* (Pc).

Strain	Selection factor			
	Incubation time (days)			
	7	14	21	28
WT	7.8 ± 5.4[a]	4.6 ± 0.6[a]	3.0 ± 0.1[a]	2.6 ± 0.2[a]
BM-65	12.6 ± 6.7[a]	6.5 ± 0.2[ab]	5.4 ± 1.6[ab]	4.7 ± 0.3[ab]
Pc	1.2 ± 0.1	1.7 ± 0.8	1.2 ± 0.5	1.1 ± 0.3

[a] Significantly higher than Pc (P<0.05).

[b] Significantly higher than WT (P<0.05).

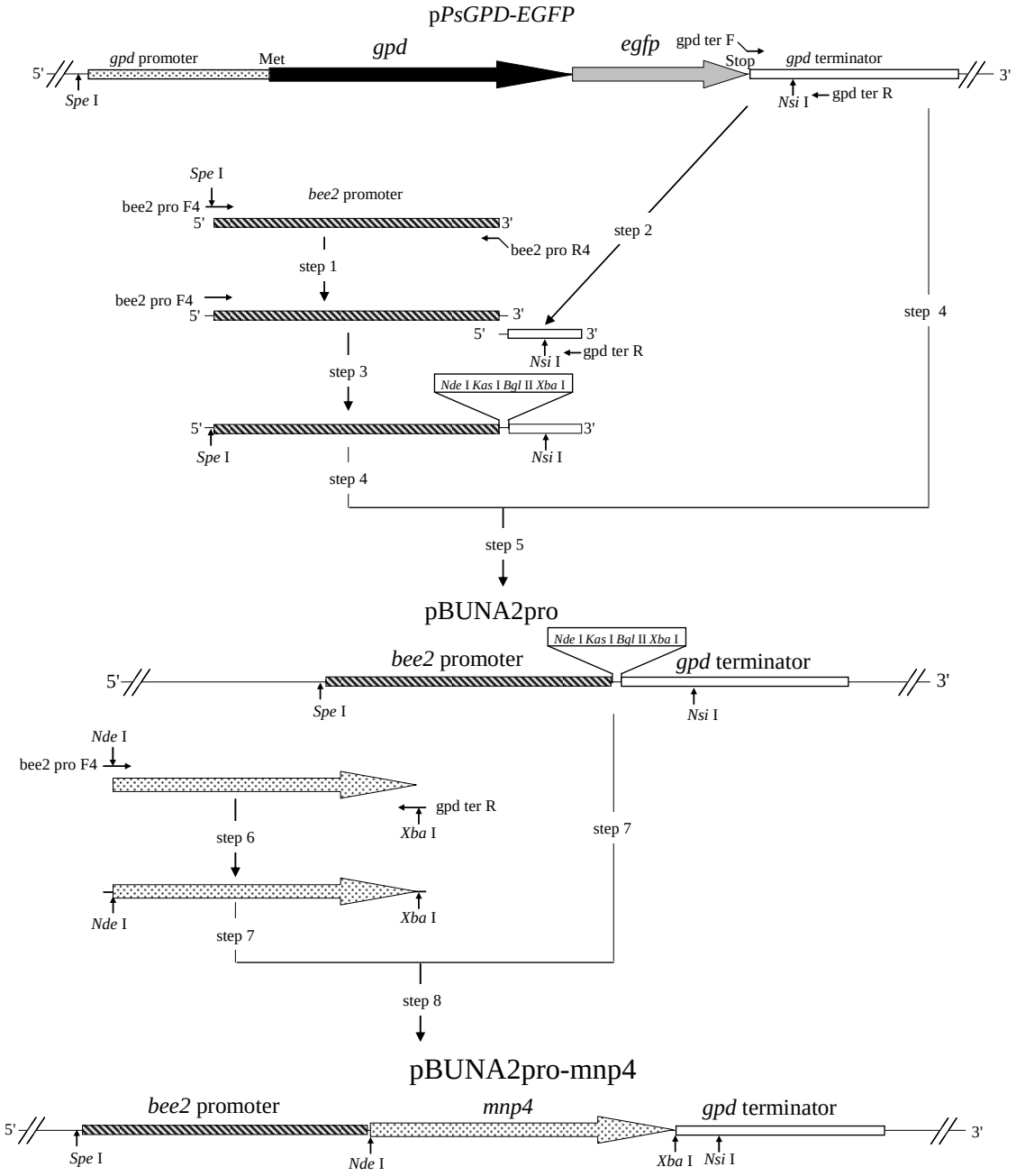


Fig. S1. Procedure used for the construction of the *mnp4* expression plasmid pBUNA2pro-mnp4. The small horizontal arrows indicate the locations and directions of the primers used for PCR amplification of the target DNA fragments. Genes are indicated by shaded boxed arrows.

Data S1.Sequence and length of Amplicon from each primer set using Real time PCR.

mnp4F2-mnp4R4 Length; 133 bp

CGACCCTTACCACTGATCCCGGTGCCTCCGAGACCCTCATCCCGCACTGCTC

GAACGGCGGCATGTCGTGCCCTGCCATCCAATTCGATGGTCCTGCTTAAGATT

ACTGCGCTTCGCTTAGTCAACAGCTAGC

mnp4F2-gpdR1 Length; 132 bp

CGACCCTTACCACTGATCCCGGTGCCTCCGAGACCCTCATCCCGCACTGCTC

GAACGGCGGCATGTCGTGCCCTGCCATCCAATTCGATGGTCCTGCTTAATCTA

GAGCGTCGCGTCACACCCATTCCCGGT

gpdF1-gpdR2 Length; 131 bp

CTCAACGGCAAGCTGACCGGCCTGTCCTTCCGTGTCCCCACCGTCGATGTCT

CCGTCGTCGACCTTGTCGTCCGTCTCGAGAAGTCCGCTTCCTACGACGAAAT

CAAGGCCGCGCTCAAGGAGGCGTCCGA

Table S1. Oligonucleotides used as primers in this study.

Primer name	Sequence (5’-3’)
BUNA2dF1	AAYCCNGTNGAYTGGAA
5phosBUNA2R[a]	CTTCGGGCTGTAGAG
BUNA2F1	TCGTCGCATCCGAGATCGTC
BUNA2F2	TGTCGAAGTAGCCCTGGAAG
BUNA2R1	CCAACCTCAGCTTCGAAGAG
BUNA2R2	CCGTGATGAAGAAGTCGAAC
BUNA2R3	GGCGTACTGCTGGAAGGTC
TAIL1	NGTCGASWGANAWGAA
TAIL2	NCAGCTWSCTNTSCTT
TAIL3	GTNCGASWCANAWGTT
TAIL4	CANGCNWSGTNTSCAA
TAIL5	WGTGNAGWANCANAGA
TAIL6	SCACNTCSTNGTNTCT
bee2proF1	AAACACCGCCCATGACAG
bee2proF2	GTTCTTCCCACCACTTTGC
bee2proF3	TGGTCTTCGGCGCGATCATC

bee2proF4	GGACTAGTTGGTCTTCGGCGCGATCATC
bee2proF5	CACACATCATCCGTCGTG
bee2proR1	CATAAGATGTCCGACGTAAG
bee2proR2	AGAGCTCCTCAATAAGACTG
bee2proR3	GGCTGTCGGGTGGTGG
bee2proR4	TCTAGAGGTACCAGATCTCATATGGGCTGTCGGGTGGTGG
gpdterF	CATATGAGATCTGGTACCTCTAGAGCGTCGCGTCACACCCATTC
gpdterR	CTACCTCCGAGCTCGCATTC
mnp4F1	CAGCCCATATGGCTTTCAGCACCCTC
mnp4F2	CGACCCTTACCACTGATCC
mnp4R1	GACGCTCTAGATTAAGCAGGACCATCGAATTG
mnp4R2	TGGAAGAGGTTTGCTTGAAG
mnp4R3	CAACGTACCGGTACGAATG
mnp4R4	GCTAGCTGTTGACTAAGCGAAG
gpdF1	CTCAACGGCAAGCTGACC
gpdR1	ACCGGGAATGGGTGTGAC
gpdR2	TCGGACGCCTCCTTGAG

H(A/T/C), N(A/T/G/C), R(A/G), S(C/G), Y(C/T), W(A/T), K(G/T)

[a] 5' end was phosphorylated.

Table S2. Peptide fragments of BUNA2 identified by a Mascot search.

m/z	Charge	Sequence	Protein name	Taxonomy
434.78	2	ALVLVPTR	ATP-dependent RNA helicase DBP9	*Ustilago maydis*
432.22	2	KSMVAGDR	Hypothetical protein MGG_04031	*Magnaporthe grisea* 70-15
541.80	2	SLGATHILDR	Hypothetical protein CC1G_12719	*Coprinopsis cinerea* okayama7#130
651.84	2	IASTWEGIQAAR	Protein of unknown function; Nqm1p	*Saccharomyces cerevisiae*
557.80	2	SVGLNPVDWK	Hypothetical protein CC1G_07820	*C. cinerea* okayama7#130

Table S3. Peptide fragments of BUNA2 deduced by *de novo* sequencing.

m/z	Charge	Mass	Amino acid sequence[a]
432.2247	2	862.4296	QFAVQDR
434.7831	2	867.5429	LAVVLEPK
441.7471	2	881.4719	GATHLLDR
511.7924	2	1021.5668	NVLPLNQPK
593.8329	2	1185.6353	VGAGVTNVAVGDK
594.6511	3	1780.9221	VEVHVFGSVQAPDKNR
629.8205	2	1257.6143	VLFQGYFDNR
662.9156	2	1323.7874	VLLVLAQAVDAGR
810.4798	2	1618.9294	VEVLPGGLAGLPAGLEK
812.9400	2	1623.8467	DLQEPGAGEVLVELR
827.4316	2	1652.8271	VEVHVFGSVQAPDNR
862.9728	2	1723.9146	ATFQQYAVVASELVAK
975.5610	2	1949.0833	GTPLSELPAAVQALTSAPVK
1002.5679	2	2003.0938	FGSLSPLLTTASPSNSALLK

[a] Leu and Ile were not distinguishable and are denoted as ‘L’

Table S4. PCR linearity and efficiency for each primer set.

	Linearity		Efficiency
	slope	r^2	
mnp4F2-mnp4R4	3.34	0.99	0.99
mnp4F2-gpdR1	3.17	0.99	1.07
gpdF1-gpdR2	3.32	0.99	1

Appendix S1.

Materials and methods

Electrophoresis, In-gel digestion and LC/MS/MS analysis

Sample precipitation and desolation

A solution containing 100 μg proteins was mixed with 1/10 volumes of trichloroacetate and was then placed on ice for 30 min before centrifugation at 15000 × *g* for 10 min at 4 °C. The supernatant was discarded and the obtained pellet was dissolved in 200 μl H_2O and 4 volumes of cold-acetone were then added. The mixture was placed at -80 °C overnight. After incubation, the suspension was centrifuged at 15000 × *g* for 10 min at 4 °C, and the supernatant was discarded. The protein pellet was dissolved in 125 μl rehydration buffer composed of Reagent3 (5 M urea, 2 M thiourea, 40 mM Tris-HCl, 2% CHAPS, 2% Sulfobetaine 3-10, and 0.2% Bio-Lyte3/10; Bio-Rad,), 50 mM dithiothreitol (DTT), and 0.001% bromophenol blue (BPB). The sample solution was subjected to two-dimensional gel electrophoresis (2-DE).

LC/MS/MS analysis

In-gel digestion was performed as previously described (Ogata et al., 2010) with the following modification: a small gel piece from the target protein spot was

washed with wash solution (30% acetonitrile (ACN), 70 mM NH_4HCO_3) for 15 min following excision from the gel.

A NanoFronteir eLD (Hitachi High-Technologies) equipped with a MonoCap for Fast-flow (50 μm I.D. × 150 mm L, GL Sciences) separation column and a MONOLITH TRAP (0.05 mm I.D. × 150 mm L; Hitachi High-Technologies) trap column was used for LC-MS/MS analysis. The mobile phase A was 98% H_2O/2% ACN with 0.3% formic acid and the mobile phase B was 2% H_2O/98% ACN with 0.3% formic acid. The flow-rate was 200 nl min^{-1}. The gradient conditions were 98% A at 0.0 min, a linear increase to 50% B from 0.0 min to 50.0 min, a linear increase to 100% B from 50.0 min to 50.1 min (0:100), a 15 min hold at 100% B, followed by a return to 98% A for a 15-min equilibration. The ESI spray potential was 1,700 V in positive-ion mode, the curtain gas flow was 0.8 l min^{-1}, and the scan mass range was m/z 50-2,000. Peak list files of the tandem mass spectrometric data were analyzed using the MASCOT on-line server (http://www.matrixscience.com/search_form_select.html) with the following search parameters: database = NCBInr; taxonomy = Fungi; enzyme = trypsin; and modifications = carbamidomethyl (C). The generated peak list files were also analyzed using the *de novo* sequencing module of Peaks Studio v5.1 (Bioinformatic Solutions) with the following analysis parameters: modifications =

carbamidomethylation, oxidation (M), pyro-glu from E, and pyro-glu from Q.

Construction of an MnP gene expression vector, co-transformation of UV-64 and screening of regenerated clones

The plasmid p*PsGPD-EGFP*, which was generated in our previous study (Yamagishi et al., 2007), was used for the construction of the expression plasmid. Primers bee2proF4 and bee2proR4 were designed to amplify the full-length sequence of the *bee2* promoter just before the first methionine codon of *bee2* and to introduce a multicloning site (NdeI-KasI-BglII-XbaI) in the 3'-terminal sequence and a SpeI site in the 5'-terminal sequence of the *bee2* promoter, respectively. These primers were used to amplify the *bee2* promoter using TaKaRa Ex Taq (step 1). Primers gpdterF and gpdterR were designed to amplify the region including the NsiI site in the *PsGPD* terminator and to add a multicloning site (NdeI-KasI-BglII-XbaI) in the 5'-terminal sequence of the *PsGPD* terminator. These primers were used to amplify the *PsGPD* terminator fragment (step 2). A mixture of the step 1 and 2 PCR products was used as template in a third_ PCR using the primers bee2proF4 and gpdterR (step 3). The amplified DNA fragment and p*PsGPD-EGFP* were digested with SpeI and NsiI (New England Biolabs) (step 4), and the digested DNA fragment was inserted into the SpeI and NsiI sites of

p*PsGPD-EGFP*, yielding plasmid pBUNA2pro (step 5). pBUNA2pro was sequenced to verify the absence of PCR errors. Primers mnp4F and mnp4R were designed to amplify the *mnp4* gene and to introduce an NdeI site just before the first methionine codon and to add an AscI site just after the stop codon, respectively (step 6). The amplified DNA fragment was digested with NdeI and XbaI (New England Biolabs) and cloned into NdeI-XbaI digested pBUNA2pro, yielding plasmid pBUNA2pro-mnp4 (step 7). pBUNA2pro-mnp4 was sequenced to verify the absence of PCR errors.

Detailed determination of ligninolytic properties

MnP activity was determined by monitoring the oxidation of 2,6-dimethoxy phenol (DMP) at 470 nm. The reaction mixture (1 ml) contained 1 mM DMP, 1 mM MnSO4, 50 mM malonate buffer (pH 4.5), and 0.2 mM hydrogen peroxide. One katal of MnP activity was defined as the amount of enzyme that oxidizes 1 mol of DMP to coerulignone (49.6 mM-1 cm-1) per second. LiP activity in the culture was determined by monitoring the oxidation of ferrocytochrome c at 550 nm. The reaction mixture (1 ml) contained 13 μM ferrocytochrome c, 20 mM succinate buffer (pH 3.0), and 0.2 mM hydrogen peroxide. One katal of LiP activity was defined as the amount of enzyme which oxidizes 1 mol of ferrocytochrome c to ferricytochrome c (19.5 mM-1 cm-1) per

second.

Transcriptional analysis of BM-65

Primer design

mnp4F2 and gpdF1 were designed striding across an intron of these encoding genes. Primers were designed to generate amplified DNA fragments with similar GC contents and of appropriate different lengths ranging between 131 and 133 bp. Sequence and length of Amplicon from each primer set were shown in Data S1.

Real time PCR Analysis

For the LightCycler reaction, a mastermix of the following reaction components was prepared to the indicated end-concentrations: 6.4 μl H_2O, 0.8 μl forward primer (0.4 μmol l^{-1}), 0.8 μl reverse primer (0.4 μmol l^{-1}), and 10 μl SYBR Premix Ex Taq GC (TaKaRa Bio). LightCycler mastermix (18 μl) and 2 μl cDNA as PCR template were then added to the LightCycler glass capillaries. The following PCR conditions were employed: initial denaturation at 95 °C for 1 min, followed by 40 cycles consisting of denaturation (95 °C, 5 s), annealing (57 °C, 10 s), and elongation (72 °C, 10 s). To confirm amplification specificity, the PCR products amplified using each primer pair were subjected to melting curve analysis using LightCycler Software

4.05 and agarose gel electrophoresis. a DNA dilution series ranging from 100 ng μl^{-1} to 1 fg μl^{-1} was used for calculating PCR linarites and efficiencies from the formula $E = 10^{1/slope}$ (Pfaffl et al., Table S4). Relative expression ratios of natural and recombinant *mnp4* were calculated using RENT 2009 software.

Reference

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res **29:** 2002–2007.