APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Transformation by complementation of a uracil auxotroph of the hyper lignin-degrading basidiomycete *Phanerochaete sordida* YK-624

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Abstract *Phanerochaete sordida* YK-624 is a hyper lignin-degrading basidiomycete possessing greater ligninolytic selectivity than either *P. chrysosporium* or *Trametes versicolor*. To construct a gene transformation system for *P. sordida* YK-624, uracil auxotrophic mutants were generated using a combination of ultraviolet (UV) radiation and 5-fluoroorotate resistance as a selection scheme. An uracil auxotrophic strain (UV-64) was transformed into a uracil prototroph using the marker plasmid p*PsURA5* containing the orotate phosphoribosyltransferase gene from *P. sordida* YK-624. This system generated approximately 50 stable transformants using 2×10^7 protoplasts. Southern blot analysis demonstrated that the transformed p*PsURA5* was ectopically integrated into the chromosomal DNA of all transformants. The enhanced green fluorescent protein (EGFP) gene was also introduced into UV-64. The transformed *EGFP* was expressed in the co-transformants driven by *P. sordida* glyceraldehyde-3-phosphate dehydrogenase gene promoter and terminator regions.

Keywords *Phanerochaete sordida* · EGFP ·
Genetic transformation · *PsURA5* · Uracil auxotroph

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Introduction

White-rot basidiomycetes are the only known microorganisms capable of degrading lignin to CO_2 and H_2O (Gold and Alic 1993). Additionally, these fungi have a superior ability to degrade complex chemical components, including toxic aromatic pollutants and industrial dyes. Lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase are involved in the lignin degradation system of white-rot basidiomycetes (Gold and Alic 1993).

Phanerochaete sordida is a widely distributed white-rot basidiomycete. Previous studies have demonstrated that the strain *P. sordida* YK-624, which was isolated from rotted wood, is excellent for degrading high-molecular-weight lignin, various kinds of environmental pollutants (e.g., polychlorinated dibenzo-*p*-dioxins), and industrial dyes (Harazono and Nakamura 2005; Hirai et al. 1994; Takada et al. 1996). Moreover, *P. sordida* YK-624 exhibits greater ligninolytic selectivity among beech woods than either *P. chrysosporium* or *Trametes versicolor* (Hirai et al. 1994). In addition, *P. sordida* YK-624 produces novel lignin peroxidases (YK-LiPs) under manganese-deficient, nitrogen-limited conditions. These YK-LiPs exhibit a higher affinity for lignin dimers and oligomers than the typical LiP from *P. chrysosporium* (Hirai et al. 2005; Sugiura et al. 2003).

In recent years, a gene transformation system for several white-rot basidiomycetes has been developed for studying and improving the lignin-degrading abilities of these fungi. Likewise, the development of a genetic transformation system for *P. sordida* YK-624 would be significant in elucidating the mechanism of its ligninolytic selectivity. It might also become possible to produce YK-LiPs constitutively by introducing YK-LiPs genes with strong and constitutive promoter sequences.

In this study, we report the development of a transformation system for *P. sordida* YK-624 using the uracil auxotrophic strain ultraviolet (UV)-64 and an isolated orotate phosphoribosyltransferase gene (*PsURA5*) from *P. sordida* YK-624. The expression of co-transformed enhanced green fluorescent protein (*EGFP*) driven by glyceraldehyde-3-phosphate dehydrogenase gene (*PsGPD*) promoter and terminator regions is also described.

Materials and methods

Strains and media

P. sordida YK-624 (ATCC 90872) was used in the present study (Hirai et al. 1994). *P. sordida* YK-624 was cultured on a complete yeast medium (CYM; yeast extract 2 g/l, polypeptone 2 g/l, glucose 20 g/l, pH 6.0) liquid or semisolid (1.5% agar) at 30°C. The uracil auxotrophic strain UV-64 was grown in CYM supplied with uracil (0.1 g/l). Protoplasts of *P. sordida* YK-624 and UV-64 were regenerated in CYM regeneration semisolid containing 1% low melting point agarose (SeaPlaque Agarose, Cambrex) and 1.0 M sucrose.

Purification of genomic DNA

Genomic DNA was prepared from a mycelium mat cultured on a cellophane membrane overlying CYM semisolid for 5 days. Two hundred milligrams of mycelium mat was ground to a fine powder using a mortar and pestle under liquid nitrogen. One milliliter of extraction buffer (Tris–HCl 200 mM, NaCl 250 mM, ethylenediaminetetraacetic acid [EDTA] 25 mM, pH 8.5) was added to the mycelium powder and mixed gently. An equal volume of phenol chloroform mixture was added, mixed gently, and centrifuged, and the aqueous phase was recovered. One unit of ribonuclease was added and incubated at 37°C for 10 min. An equal volume of chloroform was added, mixed gently, and centrifuged, and the aqueous phase was recovered. Isopropanol was added (0.54 volume), and the resultant DNA precipitate was twisted around a glass rod. The DNA precipitate was rinsed with 70% ethanol and then 100% ethanol. Finally, the DNA precipitate was dried for 15 min and rehydrated in TE solution (Tris–HCl 10 mM, EDTA 1 mM, pH 8.0).

Purification of plasmids

Plasmids for cloning and vector construction were prepared by using a QIAPrep Spin Miniprep Kit (Qiagen K.K.). Plasmids for transformation experiments were prepared by using a Quantum Prep Plasmid Midi Kit (Bio-Rad).

Purification of RNA

Total RNA of *P. sordida* YK-624 was isolated as follows. A piece of *P. sordida* YK-624 mycelium was cultured for 5 days in manganese-deficient, nitrogen-limited liquid medium to induce the expression of lignin peroxidases (Hirai et al. 2005). The cultured mycelium was recovered with a Buechner funnel and ground to a fine powder under liquid nitrogen using a mortar and pestle. Total RNA from the powder was purified by using an RNeasy Mini Kit (Qiagen K.K.).

Synthesis of cDNA

The first complementary DNA (cDNA) strand used for reverse transcriptase polymerase chain reaction (RT-PCR) was prepared using Takara RNA LA PCR kit (AMV) version 1.1 (Takara Bio). Total RNA from *P. sordida* YK-624 cultured in manganese-deficient, nitrogen-limited liquid medium was used as a template.

Procedure for cloning a genomic DNA fragment encoding the full-length PsURA5

The genomic DNA of *PsURA5* was cloned by a series of PCR procedures. Table 1 lists the primers used. Figure 1a illustrates the positions and directions of the primers in the *PsURA5* genomic DNA. The conserved region of orotate phosphoribosyltransferase was amplified using the degenerate forward primer URA5-dFA or URA5-dFG (corresponding to amino acid residues FGPAYK) and degenerate reverse primer URA5-dR (corresponding to KDHGEGG). The 3′-end region of URA5-dFA and URA5-dFG was AAA and AAG (lysine codon triplet), respectively. Degenerate PCR was performed for 35 cycles of template denaturation at 95°C for 30 s, primer annealing at 58°C for 1 min, and DNA extension at 72°C for 1 min using HotStarTaq DNA Polymerase (Qiagen K.K.). cDNA of *P. sordida* YK-624 was used as a template. A single 120-bp PCR product was strongly amplified with URA5-dFA and URA5-dR. No PCR products were amplified with URA5-dFG and URA5-dR under the same conditions. The 120-bp PCR product was cloned and sequenced. The 3′ coding region was cloned by 3′-rapid amplification of cDNA ends (3′-RACE) using two nested primers, F1 and F2. Thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) was performed to obtain the 5′ and 3′ flanking regions using genomic DNA as a template. The TAIL-PCR procedure was described previously (Yamagishi et al. 2002). Primers TAIL 1, 2, 3, 4, 5, and 6 were used as degenerate primers. Nested primers R1, R2, and R3 were used as gene-specific primers for amplifying 5′ flanking regions. Nested primers F1, F2, and F3 were used as gene-specific primers for amplifying the 3′ flanking region. Primers F4 and R4 were designed to amplify the *PsURA5* gene locus using PrimeStar

Table 1 Oligonucleotides used as primers in this study

Name	Nucleotide sequence
URA5-dFA	TTYGGNCCNGCNTAYAAA
URA5-dFG	TTYGGNCCNGCNTAYAAG
URA5-dR	CCNCCYTCNCCRTGRTCYTT
F1	GGCATCCCCTTCGCTGCTTGCACCG
F2	CGATGTTGGGTACGCGTATGACAGGAAGG
F3	ATATGGCGTCAAGGACGCGTGAGCGACTTG
F4	GTACATCGTATCTCCGTAGGTCCAGC
F5	ATGTCGGCCCTTCAGAAGTACCAGACCGA
F6	TCGACGTTCTCTTCGGTCCTGCGTACG
R1	TCCTTGCGAACGGTGTCCATCGCCTGCCT
R2	GTCCAGAACGAGGACCCGCTTGCCCTTCAC
R3	CATGACGCCACCCTCGCCGTGATCCTTCGC TTC
R4	AAACTCATCGTCTGCGGACGAATTTCA
R5	TGCACAACTCGCCATGTCATTTAT
TAIL1	NGTCGASWGANAWGAA
TAIL2	NCAGCTWSCTNTSCTT
TAIL3	GTNCGASWCANAWGTT
TAIL4	CANGCNWSGTNTSCAA
TAIL5	WGTGNAGWANCANAGA
TAIL6	SCACNTCSTNGTNTCT
URA3-dFC	CAYGTNGAYATHATHGARGAYTTC
URA3-dFT	CAYGTNGAYATHATHGARGAYTTT
URA3-dR	TCNGCRAAYTTNCKRTCYTCRAA
URA3-F1	ATGGCCCACAGGCAGACTTAC
URA3-R1	GCCTGCTACTGTTTTTATTTCGCC
GPD-F1	CTTGCTCCCAGAGTTATTCAGATTCC
GPD-F2	CGGGGCGCGATTGATTTGAGGCGA
GPD-F3	GGCGGCGGCGCGCCCTAAGCGTCGCGTC ACACCC
GPD-R1	AGAAGGCATAGGCGGTGCATGCAAACGAGC
GPD-R2	TTAGGGCGCGCCGCCGCCGAGGGCGCCGTC GACCTTCGC
GPD-R3	GGCTTTCCCGAAACCAAAGCAGTG
EGFP-F1	GGCGGCTCGGGCGGCGGCATGGTGAGCAAG
EGFP-F2	GGCCATGGCTCGGGCGGCGGCTCGGGCGG
EGFP-F3	GGGCGCGCCCATGGGCTCGGGCGGCGGCT
EGFP-F4	GACGTAAACGGCCACAAGTTCAGCGTGTCC
EGFP-R1	CGTTGGGGTCTTTGCTCAGGGCGGACTGGG
EGFP-R2	GGGCGCGCCTTACTTGTACAGCTCGTCCA
EGFP-R3	CGTTGGGGTCTTTGCTCAGGGCGGACTGGG

A/T/C H, *A/T/G/C* N, *A/G* R, *C/G* S, *A/T* W, and *C/T* Y

HS DNA polymerase (Takara Bio). The PCR product was ligated in the cloning vector pCR4Blunt-TOPO (Invitrogen) and introduced into *Escherichia coli* DH5α for sequencing. The plasmids containing *PsURA5* genomic DNA (5.0 kb) were purified by using a Quantum Prep Plasmid Midi Kit and sequenced to verify PCR errors. A plasmid with no PCR errors, designated p*PsURA5*, was used for transformation of the uracil auxotrophic strain UV-64 described later.

Procedure for cloning a genomic DNA fragment encoding the PsURA3

The conserved region of *P. sordida* YK-624 orotidine monophosphate decarboxylase (*PsURA3*) was amplified using the degenerate forward primer URA3-dFC or URA3-dFT (corresponding to amino acid residues HVDIIEDF) and degenerate reverse primer URA3-dR (corresponding to FEDRKF). The 3′-terminal regions of URA3-dFC and URA3-dFT was TTC and TTT (phenylalanine codon triplet), respectively. Degenerate PCR was performed for 35 cycles of template denaturation at 95°C for 30 s, primer annealing at 58°C for 1 min, and DNA extension at 72°C for 1 min using HotStarTaq DNA Polymerase. cDNA of *P. sordida* YK-624 was used as a template. A single 96-bp PCR product was strongly amplified using the primer set URA3-dFC and URA3-dR. No PCR products were observed using URA3-dFT and URA3-dR under the same conditions. The 96-bp PCR product was cloned and sequenced. The full-length *PsURA3* genomic DNA was cloned by the series of PCR procedures already described above for *PsURA5* (data not shown).
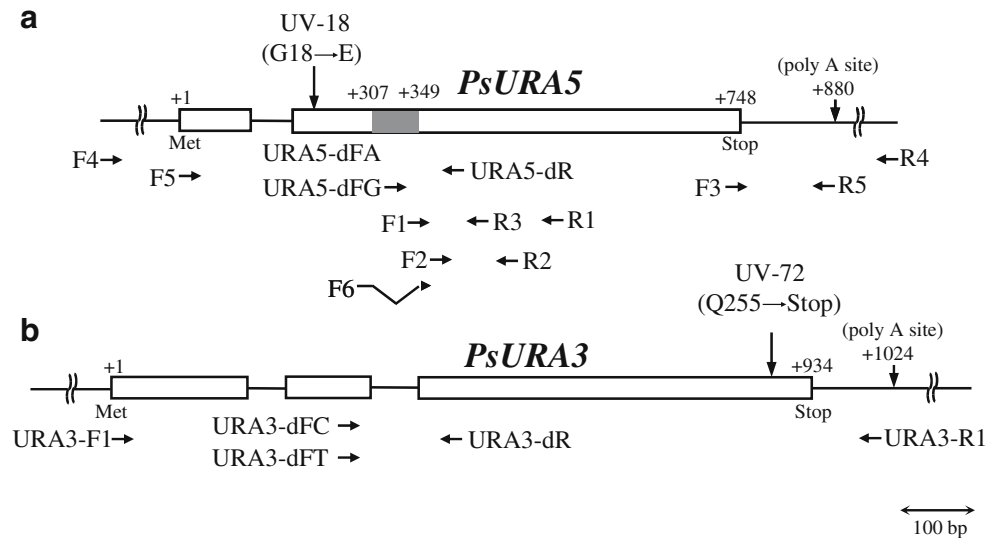
Preparation of P. sordida YK-624 protoplasts

P. sordida YK-624 was cultured at 30°C in ten conical flasks (500 ml) containing 100 ml of liquid CYM with static culture. To provide fresh mycelia for preparing protoplasts, the mycelia were homogenized with a homogenizer (Cell Master, Iuchi) at 5,000 rpm for 1 min and subcultured for a day in 20 conical flasks (500 ml). The mycelia were harvested by aspiration with a Buechner funnel. About 20 ml of wet mycelium was incubated in 50 ml of 0.75 M MgOsm (0.75 M MgSO$_4$, 20 mM 4-morpholineethanesulfonic acid [MES], pH 6.3) containing 1.5 g of Cellulase-Onozuka RS (Yakult) and 1.5 g of Lysing Enzymes L2773 (Sigma Aldrich Japan) at 30°C with gentle shaking. Some protoplasts started appearing after 3 h of shaking, and the amount of protoplast continued to increase during shaking overnight (about 18 h). The mycelium suspension was overlaid with 1.0 M SorbOsm (1.0 M sorbitol, 10 mM MES, pH 6.3) and centrifuged (4,000×g, 30 min). Protoplasts were accumulated at the interface between the two liquid phases. The protoplasts were then recovered, suspended in 1.0 M SorbOsm, and washed twice with 1.0 M SorbOsm by centrifugation (2,000×g, 15 min).

Protoplast viability test

Protoplast viability was measured as follows. After the number of protoplasts was counted with a hematocytometer, the protoplast solution was serially diluted, plated on regeneration semisolid medium, and incubated for 7 days.

Fig. 1 Structure of *PsURA5* (**a**) and *PsURA3* (**b**) genomic DNA and the locations of the primers used in this study. The *white boxes* represent coding regions of each gene. The *horizontal arrows* indicate the locations and directions of the primers mentioned under "Materials and methods." The locations of point mutations in the uracil auxotrophs UV-18 and UV-72 are also indicated. The *shaded region* in *PsURA5* indicates the region deleted (43 bp) in the uracil auxotroph UV-64

The protoplast viability rate was calculated by comparing the number of protoplasts counted using a hematocytometer with the number of regenerated clones.

Isolation of uracil auxotrophic strains derived from P. sordida YK-624

Protoplasts (10^6 cells) derived from *P. sordida* YK-624 were suspended in 1.0 M SorbOsm and radiated with a UV transilluminator (260 nm, 10 W) for 45, 60, 90, and 180 s. The distance from the UV lamp was 10 cm, at which the intensity of UV was 0.75 mW/cm^2. After irradiation, each protoplast suspension was incubated at 4°C for 18 h in darkness to prevent photoreactivation. The suspension was then regenerated with 5-fluoroorotate (5-FOA) at 0.75 g/l (Wako) and uracil at 0.1 g/l. Seven days later, 5-FOA-resistant clones were picked up and transferred to CYM semisolid with or without uracil, or with orotic acid, to verify if the clones were deficient in *PsURA5* or *PsURA3*. Three clones (UV-18, 64, and 72), which were unable to grow without uracil and with 0.5 g/l orotic acid, were identified as *PsURA5* or *PsURA3* deficient. The genomic DNA of all three clones was recovered to confirm which gene was deficient. The coding sequence of *PsURA5* or *PsURA3* was amplified with the primer set F5 and R5 for *PsURA5*, and URA3-F1 and URA3-R1 for *PsURA3*. The PCR products were purified and direct-sequenced to ascertain if *PsURA5* or *PsURA3* was mutated in each clone.

Transformation of uracil auxotrophic clone UV-64

The transformation was performed following the protocol for the transformation of the *P. chrysosporium* established by Alic et al. (1989), with some modifications. Protoplasts

of UV-64 (2×10^7 cells, 200 μl) were mixed with 120 μl of DNA solution (circular p*PsURA5*, 1.0 M SorbOsm, 40 mM CaCl$_2$) and incubated on ice for 10 min. The amount of DNA used for transformation ranged from 1.25 to 20 μg. Five micrograms of linearized p*PsURA5* digested with *Spe*I was also used for transformation (*Spe*I did not digest *PsURA5* but did digest the cloning vector pCR4Blunt-TOPO). The protoplast/DNA mixture was underlaid with 50% *w/v* polyethylene glycol (PEG; PEG4000 in 1.0 M SorbOsm, 320 μl), incubated on ice for 10 min, mixed gently, and incubated for an additional 10 min. Two hundred milliliters of regeneration medium without uracil was then added, poured into 15 petri dishes, and incubated at 30°C. After 7 to 10 days, all regenerated clones were picked up and inoculated onto CYM semisolid without uracil. The clones growing on uracil-free medium were counted 5 days later and considered as uracil prototrophic transformants. Transformation tests were repeated again to evaluate the transformation efficiency. Twelve clones transformed with 20 μg of p*PsURA5*, denoted as T1 to T12, were used for further analysis.

Southern blot analysis of exogenous PsURA5 in transformants

The genomic DNA of *P. sordida* YK-624, UV-64, and 12 transformants (T1 to T12) was purified and digested with *Kpn*I. The digested or undigested genomic DNA was electrophoresed in 0.8% agar, stained with VistraGreen (GE Healthcare) to see if it was fully degraded, and transferred to a Hybond-N$^+$ nylon membrane (GE Health-care). To prepare a hybridization probe, the primer set F2 and R5 was used for PCR amplification with p*PsURA5* as a template. The PCR product was purified by using a QIAquick PCR purification kit (Qiagen K.K.). Probe

labeling, hybridization, and signal detection were done using GeneImages random prime labeling and a CDP-star detection kit (GE Healthcare).

PCR analysis of genomic DNA from transformants

The primer set F6 and R2 was used to selectively amplify the 43 bp-deleted *PsURA5* in genomic DNA of transformants. F6 was designed to amplify the 43 bp-deleted *PsURA5*, not wild-type *PsURA5*. PCRs were carried out for 30 cycles of template denaturation at 94°C for 30 s, primer annealing at 70°C for 15 s, and DNA extension at 72°C for 1 min using HotStarTaq DNA Polymerase. The PCR products were electrophoresed in 2% agar and stained with VistraGreen.

Construction of EGFP expression plasmid

The *PsGPD* gene was cloned from *P. sordida* YK-624 genomic DNA by combining the degenerate PCR, 3′-RACE, and TAIL-PCR procedures (data not shown). Primers GPD-F1 and GPD-R1 were designed to amplify the *PsGPD* gene locus using PrimeStar HS DNA polymerase. The PCR product was ligated into the cloning vector pCR4Blunt-TOPO to construct p*PsGPD*. Primers GPD-F2, GPD-F3, GPD-R2, and GPD-R3 were designed to create an *Asc*I site just inside *PsGPD*'s stop codon by PCR mutagenesis (Step 1 and Step 2). The resultant PCR product was digested with *Sac*I and ligated into p*PsGPD* to construct p*PsGPD-Asc*I (Step 3). The *EGFP* gene was also modified to add *Asc*I sites and a long amino acid linker sequence (GSGGGSGGG). Primers EGFP-F1, EGFP-F2, and EGFP-R1 were designed to join the linker sequence just inside *EGFP*'s first methionine codon using PrimeStar HS DNA polymerase (Steps 4 and 5). Primers EGFP-F3 and EGFP-R2 were designed to add *Asc*I sites to both ends of the linker-joined *EGFP* PCR product (Step 6). The PCR product was digested with *Asc*I and ligated into p*PsGPD-Asc*I (Step 7). The resultant plasmid, p*PsGPD–EGFP*, was supposed to express the PsGPD–EGFP fusion protein linked with an amino acid linker sequence. p*PsGPD–EGFP* was purified by using a Quantum Prep Plasmid Midi Kit and used for co-transformation experiments.

Co-transformation of UV-64 with pPsURA5 and pPsGPD–EGFP gene expression plasmids

The marker plasmid p*PsURA5* (20 μg) and p*PsGPD–EGFP* (20 μg) were mixed and used for co-transformation of UV-64. Twenty-four regenerated clones were picked up and subcultured on cellophane membranes overlying uracil-free CYM semisolid for 3 days to screen the clones co-transformed with p*PsGPD–EGFP*. Pieces of the mycelium were subsequently placed in a 96-well plate with Triton-X

(0.05%) in TE and then freeze-thawed three times. The supernatants were used as templates for PCR amplification to screen for *EGFP*-positive clones. Primers EGFP-F4 and EGFP-R3 were designed to amplify the transformed *EGFP* gene. PCRs were carried out for 30 cycles of template denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, and DNA extension at 72°C for 1 min using HotStarTaq DNA Polymerase.

Measurement of *EGFP* expression intensity

To evaluate the intensity of the expression of each co-transformant, all co-transformants (E1 to E12) harboring *EGFP* genes in their genomes were cultured on cellophane membranes for 7 days. Two hundred milligrams of mycelium mat was ground to a fine powder under liquid nitrogen and homogenized with ice-cold phosphate-buffered saline with protease inhibitor cocktail P8215 (Sigma) to produce a 10% (*w/v*) homogenate. The homogenate was centrifuged at 12,000×*g* for 5 min at 4°C, and the supernatant was recovered. The relative fluorescence intensity was measured with a fluorometer (Mithras LB940, Berthold Technologies). Recombinant EGFP protein (BD Bioscience Clontech) was diluted in the range of 3 μg/ml to 0.03 μg/ml to prepare a standard curve, which was used to estimate the amount of GPD–EGFP fusion protein in each supernatant. Total protein concentration in each homogenate was measured using a Dc protein assay kit (Bio-Rad). All measurements were run in duplicate.

EGFP fluorescent imaging of co-transformant E2

The co-transformant E2 and p*PsURA5*-transformant T1 (negative control) were cultured on CYM semisolid at 30°C to observe EGFP fluorescence macroscopically. When the

Table 2 Efficiency of transformation of UV-64 with marker plasmid p*PsURA5*

Transformed plasmid	Regenerated clones (average)	Transformation efficiency	
		Marker plasmid (μg)[a]	Protoplasts (%)[b]
–	0	0	0
P*PsURA5* (1.25 μg)	9	7.2	0.000045
P*PsURA5* (2.5 μg)	29.5	11.8	0.00015
P*PsURA5* (5 μg)	37	7.4	0.00019
P*PsURA5* (10 μg)	48.5	4.8	0.00024
P*PsURA5* (20 μg)	50.5	2.5	0.00025
P*PsURA5* (5 μg; linearized)	23.5	4.7	0.00012

[a] The transformation efficiency per microgram of marker plasmid
[b] The transformation efficiency per protoplast: (regenerated clones/2× 10⁷ protoplasts)×100 (%)
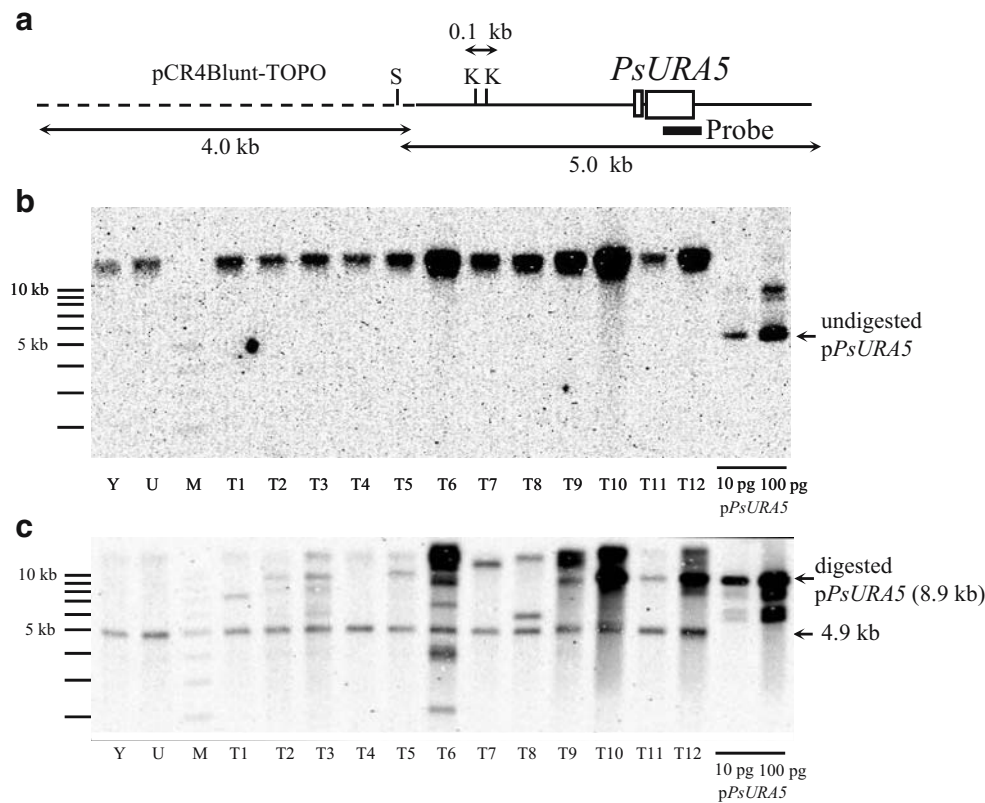
Fig. 2 Southern blot analysis of p*PsURA5* transformants. **a** Restriction map of p*PsURA5*. The cloning vector pCR4Blunt-TOPO is represented by a *dotted line*. The *heavy black line* indicates the location of the hybridization probe. *K* and *S* indicate the location of restriction enzyme sites for *Kpn*I and *Spe*I, respectively. Notice that the transformed p*PsURA5* was not linearized, but *circular*. **b** Southern blot analysis of undigested genomic DNA. The undigested genomic DNA of *P. sordida* YK-624 (*Y*), UV-64 (*U*), and 12 transformants (*T1* to *T12*) was electrophoresed, and the *PsURA5* gene was detected by Southern blot analysis. Undigested p*PsURA5* (10 or 100 pg) were also loaded. *M* indicates a 1-kb ladder size marker. **c** Southern blot analysis of *Kpn*I-digested genomic DNA. All of the genomic DNA and p*PsURA5* was digested with *Kpn*I and electrophoresed. The *PsURA5* gene was then detected by Southern blot analysis. *Kpn*I-digested p*PsURA5* (10 or 100 pg) was also loaded. *All symbols* are the same as in (**b**)

peripheral growth zone of these clones reached the edge of the 10-cm diameter petri dishes (usually 5 days), both petri dishes were illuminated at a wavelength that excites EGFP (Visi-Blue Transilluminator, UVP) and photographed through an orange filter. At the same time, a piece of mycelium was picked up from the center of each colony and mounted on slide glass for microscopic examination (Model DP12-2, Olympus).

Southern blot analysis of EGFP in co-transformants

One microgram of genomic DNA of *P. sordida* YK-624, UV-64, and 12 EGFP-transformed clones (E1 to E12) was digested with *Pst*I. The digested DNA or undigested DNA was used for Southern blot analysis. The *EGFP* gene fragment amplified by the PCR primers EGFP-F4 and EGFP-R3 was
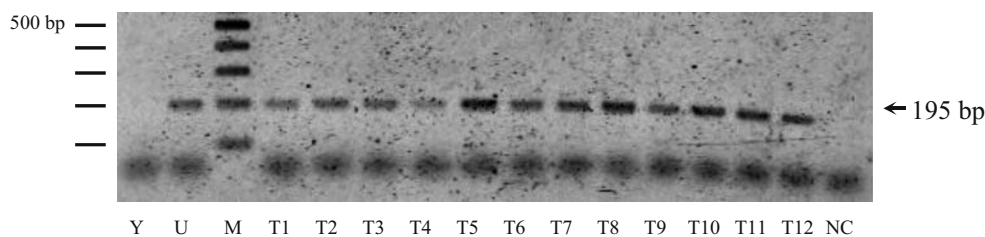
Fig. 3 PCR analysis of genomic DNA from transformants. The genomic DNA of *P. sordida* YK-624 (*Y*), UV-64 (*U*), and 12 transformants (*T1* to *T12*) was used for PCR amplification with the primer set F6 and R2 as indicated in Fig. 1a. *M* indicates a 100-bp ladder size marker. *NC* indicates negative control with no PCR templates
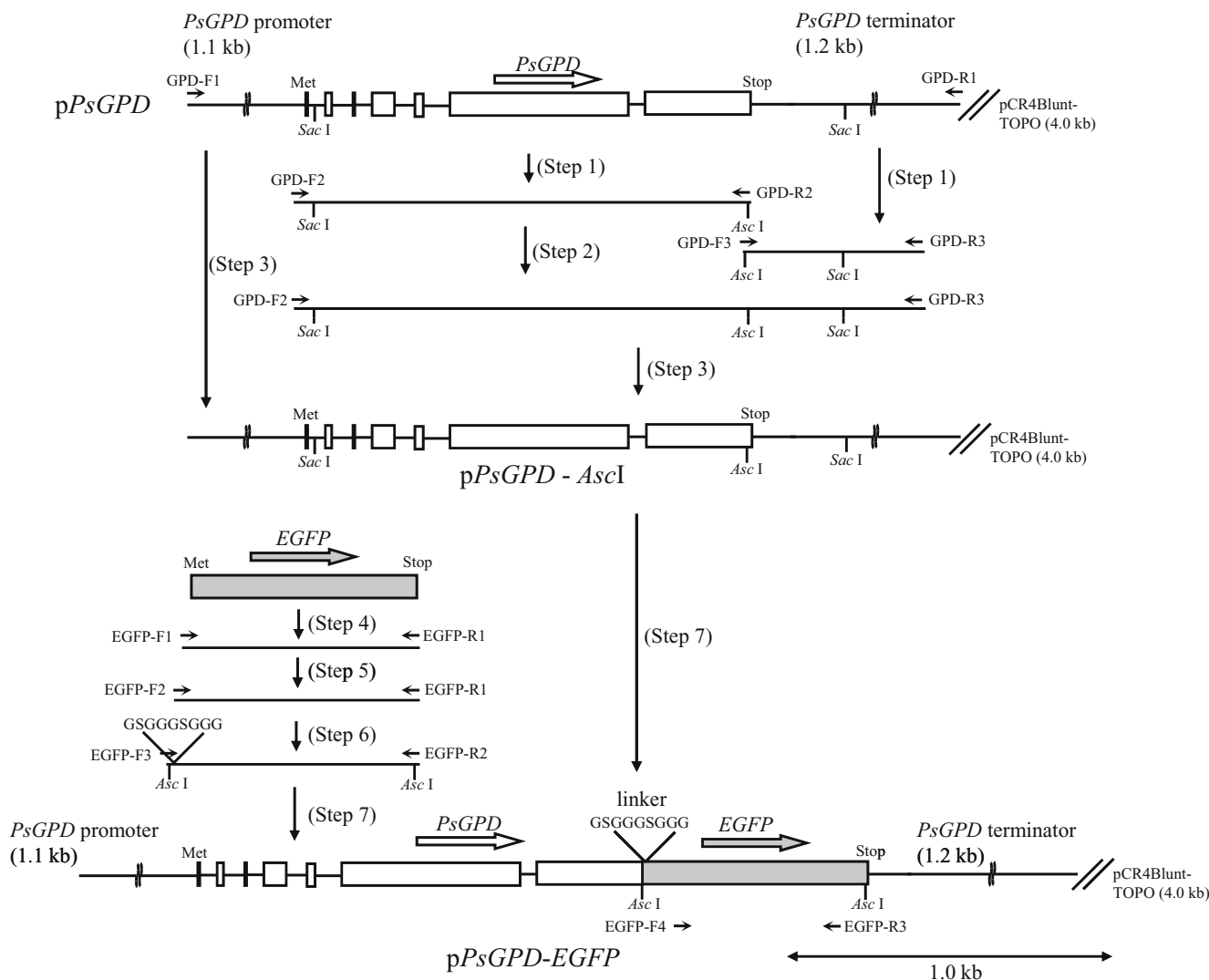
Fig. 4 Construction of *EGFP* expression plasmid p*PsGPD*–*EGFP*. The procedure used to construct p*PsGPD*–*EGFP* is described under "Materials and methods." The *horizontal arrows* indicate the locations and directions of the primers mentioned in "Materials and methods." The *thin black lines with primer arrows* indicate PCR products amplified by the primer set. *White boxes* in the *PsGPD* gene represent coding regions. Notice that the cloning vector pCR4Blunt-TOPO was not digested by either *Asc*I or *Sac*I

used as the hybridization probe. The procedures for the Southern blot analysis were the same as those for p*PsURA5*.

Assessments of genetic stability of co-transformant E2

To assess the genetic stability of the co-transformant E2, the clone was subcultured for a long period in the absence of selection pressures as follows. E2 was subcultured on CYM semisolid (containing 0.1 g/l uracil) at 30°C for 7 days. A piece of mycelium was picked up from the peripheral growth zone and subcultured on new CYM semisolid containing uracil. The old plate was denoted as generation #1 and stored at 4°C. This process was repeated five times. Finally, generations #1, #2, #3, #4, and #5 were inoculated onto cellophane membranes overlying CYM semisolid

(without uracil) in petri dishes. Transformant T1 was also cultured as a negative control. Five days later, the mycelium mat was peeled off, and the total wet weight was measured to assess the growth ability under uracil-free conditions. The EGFP fluorescent intensity of each clone was also measured as described above.

GenBank/EMBL accession numbers for genes used in this study

The GenBank/European Molecular Biology Laboratory accession numbers for *PsURA5* and *PsURA3* are AB285021 and AB285022. The accession number for *PsGPD* is AB285023. The accession number for *EGFP* purchased from BD Bioscience Clontech is U55761.

Fig. 5 Co-transformation with p*PsGPD*–*EGFP*. **a** Detection of the *EGFP* gene from 24 regenerated clones co-transformed with p*PsURA5* and p*PsGPD*–*EGFP* by PCR amplification using the primers EGFP-F4 and EGFP-R3. *M* indicates a 100-bp ladder size marker. **b** The EGFP protein content in mycelium homogenates of 12 co-transformants (*E1*–*E12*) with six transformants (*T1* to *T6*), transformed with 20 μg of p*PsURA5*, as negative controls. The EGFP protein content in each homogenate was calculated from a standard curve using known concentrations of recombinant EGFP protein, as described under "Materials and methods." All measurements were run in duplicate, and the values are represented as averages. The *dotted line* represents the average for the co-transformants (*E1*–*E12*)
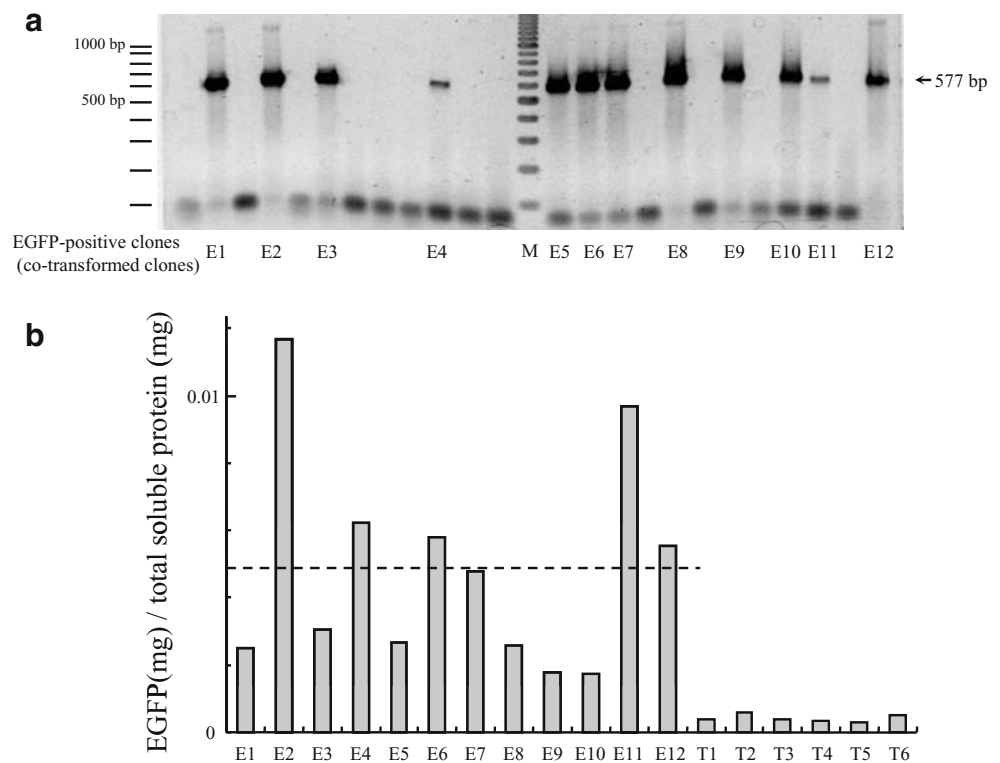
Results

### PCR-based isolation of PsURA5 and PsURA3 genes from P. sordida YK-624

As neither orotate phosphoribosyltransferase nor orotidine monophosphate decarboxylase had been reported in *P. sordida*, we began this study by isolating their genes from *P. sordida* YK-624. To design the degenerate primers for amplifying conserved regions of these genes, we referred to the genome database of *P. chrysosporium* (US Department of Energy Joint Genome Institute). In this database, scaffold 1 (459762-459887) and scaffold 38 (51064-51387) were annotated as orotate phosphoribosyltransferase (*P. chrysosporium URA5*) and orotidine monophosphate decarboxylase (*P. chrysosporium URA3*). The degenerate primer set URA5-dFA, URA5-dFG, and URA5-dR, corresponding to the conserved region of orotate phosphoribosyltransferase, was designed to perform degenerate PCR using YK-624 cDNA as a template. The PCR product was cloned and sequenced. Sequencing of five clones revealed that the PCR product consisted of a single gene, named *Ps* (*Phanerochaete sordida*) *URA5*. Similarly, the degenerate primer set URA3-dFC, URA3-dFT, and URA3-dR was used to amplify the orotidine monophosphate decarboxylase gene. Sequencing of five clones revealed that the PCR product consisted of a single gene, named *PsURA3*. The genomic

DNA fragments encoding the full length of *PsURA5* and *PsURA3* were cloned by a combination of the 3′-RACE and TAIL-PCR procedures.

Isolation of uracil auxotrophic strains from P. sordida YK-624

To create *PsURA5*- or *PsURA3*-deficient strains, *P. sordida* YK-624 was mutagenized by UV radiation. Before the mutagenesis, the susceptibility of *P. sordida* YK-624 protoplasts to UV radiation or 5-FOA was estimated. In preliminary experiments, the viability of protoplasts without UV radiation was nearly 50%. UV radiation did not affect the viability of *P. sordida* YK-624 protoplasts at durations of up to 30 and 60 s of radiation yielded about a 25% survival rate. Two minutes of radiation gave about a 10% survival rate, and no clones were regenerated after 4 min of radiation. The effect of 5-FOA on protoplast regeneration was evaluated by microscopic observation. The presence of 5-FOA in regeneration semisolid medium completely inhibited the extension of hyphae from protoplasts at 0.75 g/l in 7 days. In contrast, a majority of protoplasts extended hyphae at 0.5 g/l 5-FOA in 7 days. After the preliminary experimentation described above, each protoplast (1×10^6) was irradiated for 0, 45, 60, 90, or 180 s. All radiated protoplasts were regenerated in the presence of 5-FOA at 0.75 g/l and uracil at 0.1 g/l for
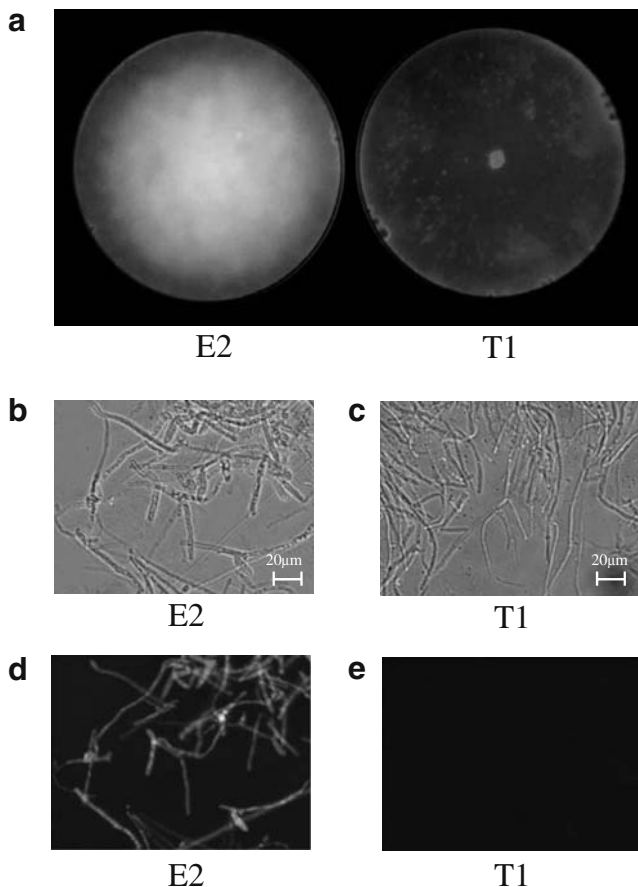
Fig. 6 EGFP fluorescent imaging of co-transformant E2. **a** Macroscopic fluorescent imaging. The co-transformant E2 and transformant T1, transformed with 20 μg of p*PsURA5*, were inoculated on a CYM semisolid without uracil and left for 5 days and then illuminated at a wavelength of 485 nm to excite EGFP with an UV-Vis illuminator. The photograph was taken with a digital camera through an orange filter. **b**–**e** Microscopic fluorescent imaging. Hyphae of E2 and T1 were microscopically observed with visible light (**b** and **c**) and EGFP excitation wavelength (**d** and **e**)

7 days. No protoplasts were regenerated without UV radiation. Thirty protoplasts were regenerated with 45 s of radiation, and 48 protoplasts were regenerated with 60 s of radiation. Two clones appeared after 90 s of radiation, and no clones appeared after 180 s of radiation. All of the regenerated clones (a total of 80) were picked up and examined for deficiencies in *PsURA5* or *PsURA3*. Orotic acid is an intermediate in the biosynthesis of uridine 5′-monophosphate (UMP) (Boeke et al. 1989). Orotidine 5-monophosphate (OMP) is synthesized from orotic acid by *PsURA5*, and OMP is decarboxylated to UMP by *PsURA3*. Therefore, uracil auxotrophic clones, which are unable to utilize orotic acid, are recognized as *PsURA5*- or *PsURA3* deficient. None of the 5-FOA-resistant clones grew without uracil at 0.1 g/l, or with orotic acid at 0.1 g/l. However, almost all of the clones were able to grow slowly with orotic acid at 0.5 g/l. This suggests that *PsURA5* or

PsURA3 does not completely lose functionality. Only three clones (UV-18, UV-64, and UV-72) did not grow with orotic acid at 0.5 g/l. PCR amplification of the *PsURA5*- and *PsURA3*-coding regions revealed that UV-18 had a missense mutation, and UV-64 had a 43-bp deletion mutation in the *PsURA5*-coding sequence (Fig. 1). In addition, UV-72 had a nonsense mutation (CAA to TAA) in the *PsURA3*-coding sequence. UV-64 was used as a gene transformation recipient clone for further experiments.

Transformation of UV-64 with a PsURA5-deficient clone

p*PsURA5*, which contained the 5.0-kb *PsURA5* genomic DNA fragment of YK-624, was used to transform UV-64 protoplasts as a marker plasmid. The protoplasts (2×10^7) were transformed with p*PsURA5*, and the clones regenerating on a uracil-free medium were enumerated (Table 2). The amount of p*PsURA5* used for transformation did not affect the transformation efficiency per protoplast in the range of 5 to 20 μg. The linearization of p*PsURA5* did not improve the transformation efficiency in comparison with a circular plasmid. The frequency of spontaneous reversion to an uracil prototroph was less than 5×10^{-8}, as no regenerated protoplasts were observed in the absence of the marker plasmid.

Southern blot analysis of genomic DNA from transformants

A Southern blot analysis was performed to confirm the presence of the marker plasmid p*PsURA5* in the chromosomal DNA of the transformant (Fig. 2). Undigested DNA samples extracted from all transformants gave high-molecular-weight signals in comparison with undigested circular p*PsURA5* (Fig. 2b). This suggests that p*PsURA5* did not exist as circular plasmid but rather was integrated into the chromosomal DNA of transformants. The band intensities of T6, T10, and T12 suggest that multiple copies of the *PsURA5* gene were integrated into the chromosomal DNA. Digestion of a DNA sample with *Kpn*I gave a single 4.9-kb band in the parent strains (*P. sordida* YK-624 and UV-64), presumably representing the endogenous *PsURA5* gene locus (Fig. 2c). *Kpn*I's digestion of DNA extracted from all transformants gave a common 4.9-kb band and band of various other sizes. The 8.9-kb band was common to about half of the transformants (T2, T3, T6, T9, T10, T11, and T12), although its intensity differed. As the 8.9-kb band is equal to p*PsURA5* digested with *Kpn*I, it presumably represents the heterologous integration of tandem multiple copies of transforming DNA. The DNA band patterns observed in T1, T5, T7, and T8 were different from each other. This suggests that p*PsURA5* was ectopically integrated into different sites of the chromosomal DNA of each transformant.
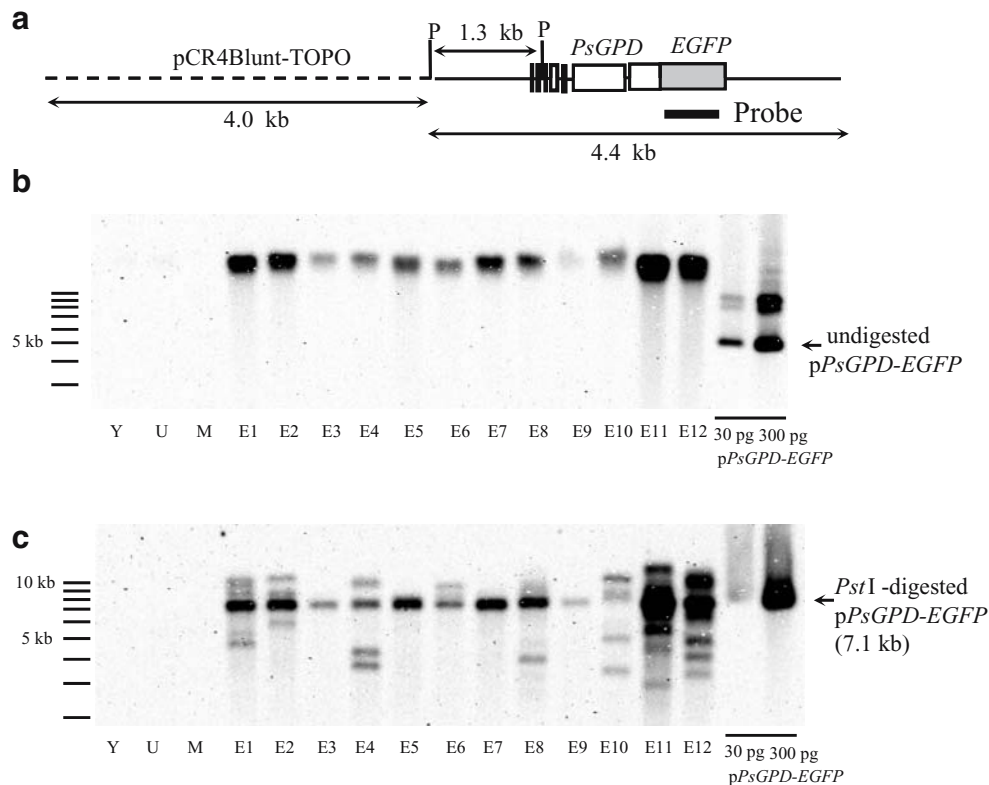
Fig. 7 Southern blot analysis of p*PsGPD*–*EGFP* co-transformants. **a** Restriction map of p*PsGPD*–*EGFP*. The cloning vector pCR4Blunt-TOPO is represented by a *dotted line*. The *heavy black line* indicates the location of the hybridization probe. *P* indicates the location of restriction enzyme sites for *Pst*I. Notice that the transformed p*PsGPD-EGFP* was not linearized, but *circular*. **b** Southern blot analysis of undigested genomic DNA. The undigested genomic DNA of *P. sordida* YK-624 (*Y*), UV-64 (*U*), and 12 co-transformants (*E1* to *E12*) was electro-phoresed, and the *EGFP* gene was detected by Southern blot analysis. Undigested p*PsGPD-EGFP* (30 or 300 pg) was also loaded. *M* indicates a 1-kb ladder size marker. **c** Southern blot analysis of *Pst*I-digested genomic DNA. All of the genomic DNA and p*PsGPD*–*EGFP* was digested with *Pst*I and electrophoresed. The *EGFP* gene was detected by Southern blot analysis. *Pst*I-digested p*PsGPD*–*EGFP* (30 or 300 pg) was also loaded. *All symbols* are as the same as in (**b**)

PCR analysis of genomic DNA from transformants

PCR amplification of *PsURA5* from the genomic DNA of transformants was performed to ascertain whether mutated endogenous *PsURA5* was disrupted in some transformants by a homologous recombination event. The primer set F6 and R2 was designed to produce a 195-bp PCR product from mutated *PsURA5* in endogenous genomic DNA of UV-64. The 3′-end of primer F6 was designed to overhang the 43-bp-deleted region of mutated *PsURA5* to prohibit the amplification of the wild-type *PsURA5* gene. The existence of 195-bp PCR products in all transformants indicates that mutated endogenous *PsURA5* was not disrupted in these clones (Fig. 3).

Co-transformation and expression of EGFP in UV-64

We isolated a *GPD* gene from *P. sordida* YK-624 to utilize its promoter and terminator regions for expressing exoge-nous genes. *EGFP* was ligated into the *GPD* as a reporter gene to ascertain if these regions could strongly express an exogenous gene. As it is recognized that endogenous introns are often essential for the successful expression of exogenous genes in basidiomycetes (Lugones et al. 1999; Ma et al. 2001; Scholtmeijer et al. 2001; Yamazaki et al. 2006), we constructed an endogenous *P. sordida* YK-624 *GPD* (including introns)–*EGFP* fusion gene and named it p*PsGPD*–*EGFP* (Fig. 4). The *EGFP* expression plasmid p*PsGPD*–*EGFP* was introduced into UV-64 using p*PsURA5* as a marker plasmid. The existence of the *EGFP* gene in each uracil prototrophic clone was confirmed by PCR using genomic DNA as a template (Fig. 5a). As a result, half (12 of 24) of the regenerated clones, denoted as E1 to E12, were co-transformed with p*PsGPD*–*EGFP*. The intensity of EGFP fluorescence in each co-transformant was measured to see if the GPD promoter and terminator regions could drive a high level of *EGFP* expression in co-transformants. The result demonstrated that the transformed *EGFP* gene was expressed in all co-transformants, although the expression level varied (Fig. 5b). The co-transformant E2
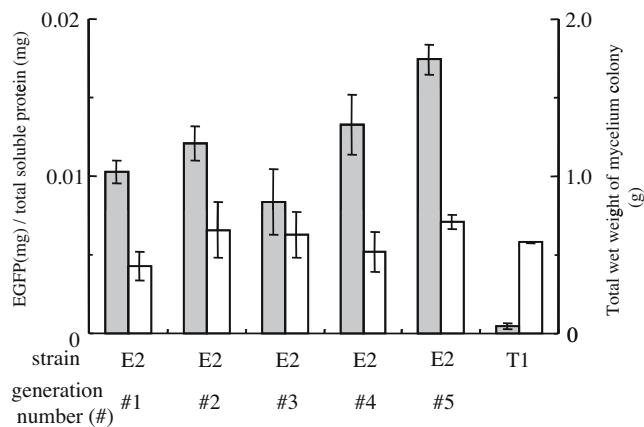
Fig. 8 Assessment of genetic stability of co-transformant E2. The EGFP protein content relative to the total amount of soluble protein appears as a *shaded bar*. The total wet weight of the mycelium mats growing on the cellophane membranes overlying the CYM semisolid (without uracil) appears as a *white bar*. All values are represented as the average±standard deviation ($n=3$)

expressed *EGFP* most strongly. The proportion of EGFP protein produced to the total amount of soluble protein indicated that 1.2% of all the soluble protein was produced by p*PsGPD*–EGFP. The expression of the GPD–EGFP fusion protein in E2 was confirmed macroscopically and microscopically. Macroscopically, EGFP fluorescence was observed throughout the co-transformant's mycelium mat (Fig. 6a). Microscopic observation also showed that the hyphae of E2 were clearly fluorescent, in contrast to the p*PsURA5*-transformed T1 (Fig. 6d and e).

Southern blot analysis of EGFP in co-transformants

A Southern blot analysis was performed to confirm the presence of the *EGFP* gene in the chromosomal DNA of co-transformants. Undigested DNA samples of all co-transformants gave high-molecular-weight signals in comparison with undigested circular p*PsGPD*–EGFP (Fig. 7b). This suggests that p*PsGPD*–EGFP did not exist as a plasmid but rather was integrated into the chromosomal DNA, as was the case with p*PsURA5*. The band intensities of E1, E2, E11, and E12 suggest that multiple copies of p*PsGPD*–EGFP were integrated into the chromosomal DNA. *Pst*I's digestion of DNA samples gave a common 7.1-kb band in all co-transformants except for E10 (Fig. 7c). As the 7.1-kb band was equal to that of p*PsGPD*–EGFP digested with *Pst*I, it presumably represented identical multiple copies of the same size as the linearized p*PsGPD*–EGFP. Other faint bands might represent the two ends of the integrated gene locus or may indicate that the integration event in the host chromosome also occurred at other sites.

Assessment of genetic stability of co-transformant E2

E2 gained two kinds of phenotypes (uracil prototroph and strong EGFP fluorescence) as a result of the co-transformation procedure. To see if these phenotypes would be lost during a long period of cultivation without any selective pressure, E2 was repeatedly subcultured on a semisolid medium containing uracil. After subculturing five times, the co-transformant did not lose the uracil prototrophic phenotype (Fig. 8). Similarly, the EGFP expression level was not significantly reduced (Fig. 8).

Discussion

Many gene transformation systems have been reported in white-rot basidiomycetes including *P. chrysosporium*, *Schizophyllum commune*, *T. versicolor*, *T. hirsuta*, *Pleurotus ostreatus*, *Lentinula edodes*, and *Pycnoporus cinnabarinus* (Akileswaran et al. 1993; Alic et al. 1989; Alves et al. 2004; Bartholomew et al. 2001; Kim et al. 1999; Munoz-Rivas et al. 1986; Sato et al. 1998; Tsukamoto et al. 2003; Yanai et al. 1996). In these reports, both auxotrophic markers (e.g., uracil, tryptophan, arginine, or adenine) and antibiotic-resistance markers (e.g., hygromycin, phleomycin, bialaphos, or carboxin) were used. At the beginning of this study, we chose to use auxotrophic markers for constructing the *P. sordida* YK-624 genetic transformation system, as antibiotic-resistance markers would be problematic due to public concerns. In contrast, self-cloning organisms, in which genes of a microorganism are cloned within the microorganism itself, are exempted from Japanese guidelines concerning safety assessments involving food microorganisms (Akada 2002; Hino 2002).

By using the uracil auxotrophic strain UV-64 and marker plasmid p*PsURA5*, we were able to construct a gene transformation system for *P. sordida* YK-624. However, the transformation rate for the protoplasts in our study was relatively low compared with that for other kinds of white-rot basidiomycetes. For example, approximately 0.3% of the viable uracil auxotrophic basidiospores were transformed with the marker gene *ura11* in *P. chrysosporium* (Akileswaran et al. 1993). In *T. hirsuta*, approximately 0.25% of the viable arginine auxotrophic protoplasts (derived from oidia) were stably transformed with the marker gene *arg1* (Tsukamoto et al. 2003). As we had not optimized the concentration of certain components (proto-plasts, calcium, and PEG) that affect DNA uptake in protoplasts, the test could be refined to increase the transformation efficiency for *P. sordida* YK-624. It has been reported that a linearized marker plasmid, p*ADE2*, resulted in a higher frequency of transformation than a

circular plasmid (Alic et al. 1989). However, linearized p*PsURA5* did not improve the transformation efficiency in this study as it did for *C. hirsutus* (Tsukamoto et al. 2003).

A Southern blot analysis of uracil prototrophic transformants revealed that p*PsURA5* was integrated into the chromosomal DNA of transformants rather than being carried in an autonomous form. This is a common event reported in many basidiomycetes including *P. chrysosporium* (Akileswaran et al. 1993; Alic et al. 1989). The existence of an 8.9-kb band, corresponding to *Kpn*I-digested p*PsURA5*, also suggests that multiple copies of linearized p*PsURA5* were integrated into one site of chromosomal DNA in about half of the transformants (Fig. 2c). This phenomenon was also reported in transformation systems for *P. chrysosporium* (Alic et al. 1989; Akileswaran et al. 1993). A multiple integration event was also observed in the *EGFP* co-transformation experiment. Characteristically, a common 7.1-kb band was observed in all of the clones except E10 (Fig. 7c). As the co-transformants were regenerated in 15 petri dishes after the transformation procedures, it is unlikely that the same regenerated protoplast clones were picked more than once. The differences in band pattern and density indicate that these co-transformants were derived from independently regenerated protoplasts. It is unclear why a multiple-integration event occurred in a large proportion of co-transformants as compared to the p*PsURA5*-transformed clones.

The intensity of fluorescence differed considerably among the co-transformants. A similar phenomenon was reported previously when *EGFP* was expressed in *P. chrysosporium* (Ma et al. 2001). A comparison of the EGFP expression level of each co-transformant (Fig. 5b) and the Southern blot analysis of the *EGFP* gene (Fig. 7) suggest that there was some correlation between the copy number of the introduced *EGFP* gene and the EGFP fluorescence of the co-transformants. For example, co-transformants E1, E2, E11, and E12 had more *EGFP* gene copies than other clones. E2 exhibited the strongest EGFP fluorescence, and the fluorescent intensities of E11 and E12 were also stronger than average. However, the fluorescent intensity of E1 was very weak, although the copy number of the *EGFP* gene was similar to that in E2. In this case, the integrated positions in the chromosomal DNA might affect the gene expression levels, which is recognized as a positional effect. A relationship was previously reported between the copy number of an integrated β-glucuronidase (GUS) gene and GUS activity (Yanai et al. 1996).

We chose the *GPD* promoter to express *EGFP*, as *GPD* is expressed strongly and stably in many species and has been used for endogenous and exogenous gene expression experiments on many kinds of white-rot basidiomycetes (Alves et al. 2004; Hirano et al. 2000; Irie et al. 2001; Kajita et al. 2004; Kuo et al. 2004; Li et al. 2000; Ma et al.

2001; Mayfield et al. 1994; Schuren et al. 1993; Schuren and Wessels 1994; Sollewijn Gelpke et al. 1999). In this study, the *GPD* promoter derived from YK-624 genomic DNA was able to produce EGFP protein at levels equivalent to up to 1.2% of all soluble protein. This suggests that the *P. sordida* YK-624 *GPD* promoter could facilitate high-level production of YK-LiPs, thus improving the high-molecular lignin degradation activity of *P. sordida* YK-624.

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