FULL PAPER

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DNA-mediated transformation system in a bipolar basidiomycete, *Pholiota microspora* (*P. nameko*)

Abstract We cloned a gene encoding the succinate dehydrogenase iron-sulfur protein subunit (*sip*) from a bipolar mushroom, *Pholiota microspora*, and introduced a point mutation that confers carboxin resistance into this gene. Using this homologous selective marker and also a heterologous drug selective marker, the hygromycin B phosphotransferase gene (*hph*), we successfully constructed a DNA-mediated transformation system in *P. microspora*. Both these selection markers have high transformation efficiency: the efficiency of carboxin resistance transformation was about 88.8 transformants/μg pMBsip2 DNA using 5×10^6 protoplasts in regeneration plates containing 1.0 μg/ml carboxin, and the efficiency of hygromycin B resistance transformation was about 122.4 transformants/μg pMBhph1 DNA using 5×10^6 protoplasts in regeneration plates containing 150 μg/ml hygromycin B. Southern hybridization analysis showed that the introduced sequence (mutant *sip* or *hph*) was integrated into the chromosomal DNA in these transformants with a copy number of one or more.

Key words Heterologous selective marker · Homologous selective marker · Hygromycin B phosphotransferase gene · Iron-sulfur protein subunit · *Pholiota microspora*

Introduction

Pholiota microspora (Berk.) Sacc. [*P. nameko* (T. Ito) S. Ito & Imai], a wood-decaying homobasidiomycete, is a major cultivated mushroom species in China and Japan. It produces an economically important edible mushroom known as "nameko" in Japan. As a consequence of recent advances in bottle cultivation technology in Japan, this mushroom is now available in the Japanese domestic market throughout the year. However, in the commercial spawn of this mushroom, the dikaryotic mycelia can sometimes produce monokaryotic hyphal cells, which can lead to very poor production or a nearly total lack of fruiting when using inferior strains (Babasaki et al. 2003). It is possible that this phenomenon is related to the mating system of this fungus.

Pholiota microspora has a bipolar mating-type system and carries a bipolar *A* mating-type locus (Ratanatragooldacha et al. 2002). The composition of the *A* mating-type region in this species has been recently described by Aimi et al. (2005), who characterized the genes encoding the homeodomain protein *A4-hox1* and the pheromone receptor *A4-rcb1*. These proteins are putative homologues of the HD1 and Rcb3 protein genes, respectively, of the tetrapolar basidiomycete *Coprinopsis cinerea* (Schaeff.) Redhead, Vilgalys & Moncalvo (Badrane and May 1999; Halsall et al. 2000). Following the work of Aimi et al., the genomic structure of the *P. microspora A* mating-type locus was analyzed and a single pair of homeodomain protein genes was found in the region (Yi et al. 2009). However, the functions of the *P. microspora A* mating-type locus in vivo have yet to be investigated as no transformation system has been designed for this species.

DNA-mediated transformation is a powerful tool for molecular analysis of fungal mating genes. Along with auxotrophic selective markers (Binninger et al. 1987), dominant drug resistance markers are very effective tools for constructing a DNA-mediated transformation system in fungi. The hygromycin B phosphotransferase gene (*hph*) from *Escherichia coli* (Migula) Castellani and Chalmers is a dominant selectable marker for antibiotic resistance that can be fused to a homologous promoter before transformation. This marker has been used successfully in transforming at least seven basidiomycetes, including two plant pathogenic smut fungi, *Ustilago maydis* (DC.) Corola (Wang et al. 1988) and *Ustilago violacea* (Pers.) Rouss. (Bej and Perlin 1989); an ectomycorrhizal fungus, *Laccaria laccata* (Scop.: Fr.) Berk. & Broome (Barrett et al. 1990); and four

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edible fungi, *Pleurotus ostreatus* (Jacq.: Fr.) Kummer (Ming et al. 1992; Li et al. 2006), *Agaricus bisporus* (Large) Sing (Rhee et al. 1996), *Lentinus edodes* (Berk.) Pegler (Hirano et al. 2000), and *Ganoderma lucidum* (Curtis) P. Karst. (Li et al. 2004). Recently, a homologous drug-resistant marker that confers dominant resistance to the systemic fungicide carboxin has been used in *P. ostreatus* (Honda et al. 2000) and *L. edodes* (Irie et al. 2003). The marker encodes a mutant iron-sulfur protein (Ip) subunit of succinate dehydrogenase.

To investigate whether only homeodomain proteins control mating and clamp cell formation in *P. microspora*, we constructed a transformation system in *P. microspora* using a heterologous and a homologous selective marker, respectively.

Materials and methods

Fungal strain

Monokaryons of *P. microspora* were obtained by monospore isolation from the fruit bodies of various wild strains (Masuda et al. 1995). Auxotrophic mutant monokaryon of *P. microspora* NGW19-6 (*A4,pdx1*) were derived from wild monokaryotic strain NGW19 (*A4*) and stocked in our laboratory.

DNA preparation

To collect carboxin-resistant (Cbx^r) transformants, five mycelial agar blocks ($5 \times 5 \times 5$ mm^3) cut from an MYG plate (glucose 2%, malt extract 0.5%, yeast extract 0.5%, agar 1.5%, pH 5.6) were transferred to 10 ml liquid MYG medium (glucose 2%, malt extract 0.5%, yeast extract 0.5%, pH 5.6) containing 2.0 µg/ml carboxin in a 100-ml Erlenmeyer flask and grown at 25°C without shaking for 2 weeks. To collect hygromycin-resistant (Hyg^r) transformants, 10 mycelial agar blocks ($5 \times 5 \times 5$ mm^3) cut from an MYG plate were transferred to 5 ml liquid MYG medium containing 200 µg/ml hygromycin in 100-ml Erlenmeyer flasks and grown at 25°C without shaking for 3 weeks. Mycelia were then harvested by filtration, frozen in liquid nitrogen, and ground to a fine powder using a mortar and pestle. Genomic DNA was extracted according to the method described by Dellaporta et al. (1983).

Amplification of succinate dehydrogenase Ip subunit gene (*sip*) of *P. microspora*

All amplified DNA fragments were subcloned into the pT7Blue (R) T-vector (Novagen, Madison, WI, USA) and then sequenced. Primers used are shown in Table 1. Initially, degenerate polymerase chain reaction (PCR) of the succinate dehydrogenase Ip subunit gene (*sip*) from the

Table 1. Primers used for identification of the *sip* and *hph* sequences

Primer	Sequence	Remark
SDIpF2	5′-ATHAARATHAARAAYGARAT-3′	Used for initial amplification
SDIpR	5′-TCRTCYTGRTTCCACCARTA-3′	
IpUp2	5′-CCCTCTTCCACACAGTGAGTACCA-3′	
IpUp1	5′-CTGCAGAATCGATCGCGATGCAAC-3′	Used for cassette PCR
IpDn2	5′-GATCGATTCTGCAGAGGCAAGCGA-3′	
IpDn1	5′-GATTTTCGCGTTCTTGGTTGCATC-3′	
CaPrC1	5′-TCGTTAGAACGCGTAATACGACTCA-3′	Used for cassette PCR
CaPrC2	5′-CGTAATACGACTCACTATAGGGAGA-3′	
Ip-d1F	5′-CTTACAAACACTGCCGCCA-3′	Used for amplification of the complete *sip* gene and the flanking region
Ip-d1R	5′-TCGACGCAGATGGCACT-3′	
Ip-d2F	5′-ACTCTGGTCGACGTCA-3′	
Ip-d2R	5′-GTCCCACAACCATTCT-3′	
Ip-d3F	5′-ACTTTCCGCAATCCTC-3′	
Ip-d3R	5′-TTTACGACGGTCTTCTG-3′	
Ip-d4F	5′-CAGAACTCTAGCCCACC-3′	Used for direct sequencing
Ip-d4R	5′-GTCGCGAGAATGGAT-3′	
Ip-d5F	5′-ACGTTGCATGTTGGAC-3′	
Ip-d5R	5′-TTGACAAGGCAGAACC-3′	
Ip-1R	5′-ACGTTCAAGCGCTCACT-3′	
Ip-cbxR	5′-ATGTACCGTTGTCTTACTATCTTCA-3′	Used for site-directed mutagenesis; the underlines showed the introduced mutant site
Ip-cbxF	5′-TTGAAGATAGTAAGACAACGGTACA-3′	
Ip-pro-XhoI	5′-TGGCGGCCTCGAGTGGCAGTGAGGAGGA-3′	Used for amplification of the promoter and terminator regions of *sip*, including pT7 blue T-vector
Ip-ter-BglII	5′-ACCGACTAAGATCTCAGTCTTCTC-3′	
pER8-XhoI	5′-CGGATCCTCGAGAATGAAAAAGCCTGAACTCACCGCGACG-3′	
pER8-BglII	5′-TTGAAAGATCTCTATTCCTTTGCCCTCGGACGAGTGCTGG-3′	Used for amplification of the *hph* gene
Hyg-F3	5′-GGGAATTCAGCGAGAGCCTG-3′	
Hyg-R3	5′-CGAAATTGCCGTCAACCAAGC-3′	

PCR, polymerase chain reaction

NGW19-6 strain of *P. microspora* was performed using primers SDIpF2 and SDIpR. The SDIpF2 and SDIpR primers were designed based on the amino acid sequences IKIKNEI and YWWNQDE, respectively, which are conserved in the *sip* of *P. ostreatus*, *L. edodes*, *A. bisporus*, and *U. maydis* (Keon et al. 1991; Irie et al. 1998; De Groot et al. 1999; Irie et al. 2003). PCR was carried out in 100-μl volumes containing 100 ng extracted genomic DNA, 100 pmol each primer, dNTPs at a final concentration of 0.2 mM, 1× PCR buffer, and 2.5 U Ex Taq polymerase (TaKaRa Bio, Shiga, Japan). Amplification parameters were as follows: an initial denaturation step at 95°C for 3 min; followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 2 min, extension at 72°C for 30 s; and a final extension at 72°C for 10 min. An approximately 500-bp DNA fragment was obtained. To obtain the complete sequence of the *sip* gene, two primers sets (IPup1/IPup2, IPdn1/IPdn2), which were designed based on the partial sequence acquired, were employed for cassette PCR. Template DNA for cassette PCR was prepared using a TaKaRa LA PCR In vitro Cloning kit (TaKaRa Bio) according to the manufacturer's instructions. Genomic DNA from *P. microspora* was digested with restriction endonucleases (*Bam*HI and *Sal*I, respectively), ligated with a nucleotide linker, and used as a template for PCR. We cloned and sequenced an approximately 1.6-kbp PCR product from IPup2 into a *Bam*HI site, including the 5′-untranslated region, and a 1.9-kbp product from IPdn2 into a *Sal*I site, including the 3′-untranslated region.

To amplify the whole genomic clone of the *sip* gene, oligonucleotide primers Ip-d1F and Ip-d1R were designed based on the sequence of DNA fragments obtained by cassette PCR. Thermal cycling parameters were as follows: initial denaturation at 95°C for 4 min; followed by 30 cycles of 95°C for 30 s, 64°C for 30 s, 72°C for 3.5 min; and a final extension at 72°C for 10 min. The amplified DNA fragment was purified using a QIAquick PCR Purification kit (Qiagen, Tokyo, Japan), then subcloned into the pT7Blue (R) T-vector (Novagen) to make plasmid pMBsip1. The purified fragment was used as a DNA template for direct sequencing with oligonucleotide primers (see Table 1).

Site-directed mutagenesis and construction of the vector plasmid

A point mutation (CAT to CTT) that causes an amino acid substitution (His240 to Leu) was introduced into the *sip* gene of *P. microspora* NGW19-6 strain. First, a 2262-bp DNA fragment containing the *sip* promoter and coding sequence was amplified using primers Ip-d1F and Ip-cbxF (5′-TTGAAGATAGTA<u>A</u>GACAACGGTACA-3′, where <u>A</u> indicates the site of base substitution). A 969-bp DNA fragment including the *sip* coding and terminator sequence was amplified using primers Ip-1R and Ip-cbxR (5′-ATG-TACCGTTGTC<u>T</u>TAACTATCTTC-3′, where <u>T</u> indicates the site of base substitution). The template DNA used for amplification of the two fragments was genomic DNA from lyophilized mycelia of *P. microspora* strain NGW19-6.

Thermal cycling parameters for the former fragment were as follows: initial denaturation at 94°C for 3 min; followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1.5 min; and a final extension at 72°C for 10 min. The amplification conditions of the latter fragment were as follows: initial denaturation at 94°C for 3 min; followed by 35 cycles of 94°C for 30 s, 59°C for 30 s, 72°C for 2.5 min; and a final extension at 72°C for 10 min. DNA fragments were separated on a 0.8% agarose gel and purified. Subsequently, the two fragments were combined by PCR to form one 3.2-kb fragment using Ip-d1F and Ip-d1R primers and the two purified fragments as templates. The amplification was initiated with a 3-min denaturation at 94°C, followed by 30 cycles of 94°C for 30 s, 64°C for 30 s, and 72°C for 3.5 min. The PCR product was subcloned into the pT7Blue (R) T-vector and sequenced to confirm the base substitution. The constructed plasmid was designated pMBsip2 (Fig. 1).

Construction of a plasmid vector containing the *hph* gene

To express the *hph* gene efficiently in *P. microspora*, we used the *sip* gene promoter and terminator. First, the *hph* gene was amplified using pER8-*Xho*I and pER8-*Bgl*II primers and a plant transforming plasmid vector (pER8; Zuo et al. 2000), which was kindly provided by Dr. Hiroyuki Kaminaka (Tottori University, Japan), as a template. Thermal cycling parameters were as follows: initial denaturation at 94°C for 4 min; followed by 35 cycles of 94°C for 30 s, 68°C for 1 min; and a final extension at 72°C for 10 min. The amplified fragment was digested with *Xho*I and *Bgl*II. Second, the fragment containing the pT7Blue (R) T-vector (Novagen) and the *sip* promoter and terminator was amplified using Ip-pro-*Xho*I and Ip-ter-*Bgl*II primers and pMBsip1 as a template. The amplification conditions were as follows: initial denaturation at 94°C for 3 min; followed by 35 cycles of 94°C for 30 s, 58°C for 1 min, 72°C for 5 min; and a final extension at 72°C for 10 min. The PCR product was also digested with *Xho*I and *Bgl*II. Then, these two
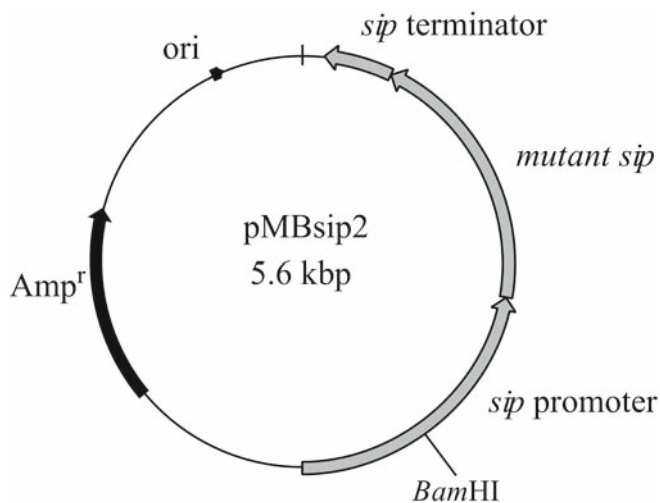

Fig. 1. Physical map of plasmid pMBsip2. The *Bam*HI recognition site is shown. *Arrows* indicate direction of transcription
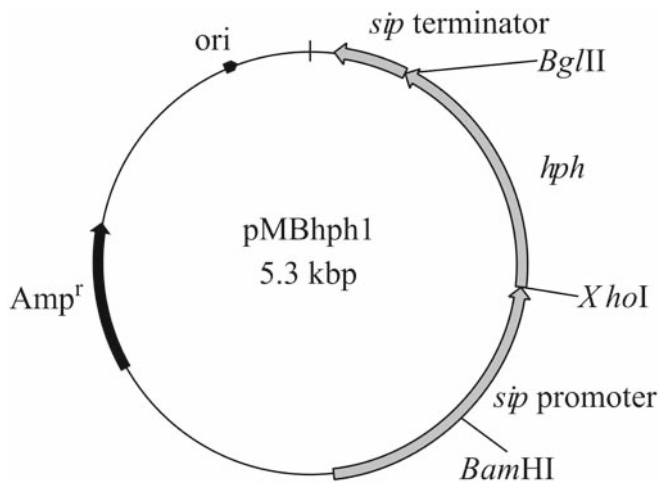
Fig. 2. Physical map of plasmid pMBhph1. The *Bgl*II, *Xho*I, and *Bam*HI recognition sites are shown. *Arrows* indicate direction of transcription

digested fragments were ligated to form plasmid pMBhph1 (Fig. 2).

Transformation

DNA-mediated transformation was carried out according to the method described by Binniger et al. (1987) and Honda et al. (2000). Briefly, to collect oidia of *P. microspora*, an MYG plate was inoculated with the NGW19-6 strain and incubated at 25°C for 2 weeks. Five agar blocks ($2\times 2\ mm^2$) cut from the plate were inoculated to 100-ml Erlenmeyer flasks containing 10 ml MYG liquid medium and incubated at 25°C for 2 weeks without shaking (if incubated for more than 2 weeks, the germination rate of oidia is low). The culture broth and mycelia mats from five flasks were transferred to 50-ml polypropylene conical tubes and shaken vigorously by hand. Culture broth was filtered through a stainless steel net and then 3G-1 glass filters, and oidia were collected by centrifugation (945 *g* for 10 min). The harvested oidia were inoculated into 100 ml MYG liquid medium in Sakaguchi flasks and incubated at 25°C for 18–24 h with shaking at 140 rpm for optimal germination (the germination rate will be low if the concentration of oidia collected is too high). The germinated oidia were collected by centrifugation (4°C, 945 *g* for 10 min); washed with sterilized distilled water and MM buffer (0.55 M mannitol, 50 mM maleic acid, pH 5.5); suspended in 3 ml MM buffer containing 2% lywallzyme (Guangdong Institute of Microbiology, Guangdong, China); and incubated at 30°C for 3 h with gentle shaking at 30-min intervals to release the protoplasts into suspension. Protoplasts were filtered through a 3G-1 glass filter into 15-ml polypropylene conical tubes, centrifuged (4°C, 340 *g* for 5 min), and washed with MMC buffer (0.55 M mannitol, 50 mM maleic acid, 50 mM $CaCl_2$, pH 5.5). Approximately 5×10^6 protoplasts were suspended in 50 µl MMC buffer, and then 20 µg plasmid (pMBsip2 or pMBhph1) and 12.5 µl PEG buffer (25% PEG4000, 10 mM Tris-HCl, 25 mM $CaCl_2$, pH 7.5) were

added. The mixtures were placed on ice for 10 min, then 500 µl PEG buffer was added. After the mixtures were kept at room temperature for 5 min, 1 ml MMC buffer and 1 ml SMYM medium (1% sucrose, 1% malt extract, 0.4% yeast extract, 0.55 M mannitol, pH 5.6) without agarose were added. Subsequently, the protoplasts were incubated at 25°C for 18–24 h, then mixed with 3 ml SMYM medium containing 0.7% agar, which was kept at 50°C, and the selective drug (three concentrations of carboxin: 0.5, 1.0, 2.0 µg/ml; four concentrations of hygromycin B: 150, 200, 300, 400 µg/ml). The mixtures were poured onto SMYM agar medium (1% sucrose, 1% malt extract, 0.4% yeast extract, 0.55 M mannitol, 1.5% agar, pH 5.6) containing the selective drug at each concentration. The plates were incubated at 25°C for 5–7 days. Colonies that appeared were subcultured individually onto fresh MYG plates containing the selective drug (for the Cbx^r transformants, the concentration of carboxin was 2 µg/ml; for the Hyg^r transformants, the concentration of hygromycin B was 200 µg/ml). Synchronous transformation experiments using pMBsip2 and pMBhph1 were performed three times, and there were three replicates for each concentration of drug. In each round of experiments, a control without the joining plasmid was used for every drug concentration.

The number of regenerating Cbx^r and Hyg^r colonies in the plates was calculated on the third day and a week later after the colonies appeared, respectively.

Southern hybridization

Southern hybridization analysis of the transformants was performed to analyze integration of the transforming DNA. Genomic DNA (0.3–0.5 µg) from the Cbx^r and Hyg^r transformants was digested for 5 h at 37°C in a 500-µl reaction mixture containing 20 units *Bam*HI in buffer supplied by the manufacturer (Toyobo, Osaka, Japan). There is a *Bam*HI site in *sip* promoter region, and not in *sip* terminator region, *sip* coding region, *hph* gene. The digested fragments were concentrated, and then separated using 1.0% agarose gels and blotted onto nylon membrane (Hybond-N+; Amersham Biosciences, London, UK). DNA hybridization probes were labeled and detected using DIG-High Prime DNA Labeling and Detection kits (Roche Diagnostics, Tokyo, Japan). We used nested PCR to make the probe. For the Cbx^r transformants, we amplified a partial pMBsip1 sequence using primers Ip-d4R and Ip-d2F. For the Hyg^r transformants, we amplified a partial pMBhph1 sequence using primers Hyg-F3 and Hyg-R3.

Results and discussion

Nucleotide and deduced amino acid sequence of *sip*

To investigate the genomic structure around the *sip* gene, which encodes the *sip* in *P. microspora*, a 3206-bp sequence around *sip* was amplified and sequenced. The sequence appears in the DDBJ/EMBL/GenBank databases with the

accession number AB455529. The length of the coding region (from ATG to the stop codon) is 1162 bp. The gene encodes a putative protein of 271 amino acids. The locations of exons and introns in the gene were deduced on the basis of interruptions in the amino acid sequence that were homologous to those in the *sip* of *L. edodes* (Irie et al. 2003), as well as from the sites of consensus sequences of conserved 5′- and 3′-splice sites of introns. All the introns started with GT and ended with AG. The coding region was split into seven exons by six introns. A BLASTX homology search (Altschul et al. 1997) was performed for the deduced amino acid sequences of the DNA fragment using the website http://www.ddbj.nig.ac.jp/E-mail/homology-j.html. The deduced protein sequence of the homeodomain in *sip* has 84% amino acid identity and 91% similarity with the Sdc-ip protein of *L. edodes* (AB092822) and 77% identity and 87% similarity with the Sdi protein of *P. ostreatus* (AB007361). We next examined the predicted protein sorting signals. The program PSORTII (Nakai and Kanehisa 1992) predicted that the Sip protein was a mitochondrial inner membrane protein (60.9% probability) and that the cleavage site for the mitochondrial sequence was between Ser26 and Gln27.

Carboxin resistance transformation

Three concentrations of carboxin (0.5 µg/ml, 1.0 µg/ml, and 2.0 µg/ml) were used in the regeneration plates for the carboxin resistance transformants. The transformation efficiency was different for plates with each of the three concentrations, and no transformants were detected in the control (TE buffer) plates (Table 2). The regeneration plates containing 1.0 µg/ml carboxin had more transformants than the others, with an average transformation efficiency of 88.8 Cbx^r colonies/µg plasmid DNA using 4.5×10^6 viable protoplasts. We randomly chose a host strain colony and six transformants picked from the 1.0-µg/ml carboxin regeneration plates for Southern hybridization analysis.

The band size corresponding to the endogenous *sip* sequence of the host strain was 2.8 kbp (Fig. 3). Several hybridization signals of various sizes were observed for the transformants, in addition to the hybridization signal of the wild-type gene. Some hybridization signals were lighter compared with other bands in the same lanes. For example, transformants Cp1–3 and Cp1–6 had an additional band located just below the host band. These two bands might be partial DNA fragments of *sip* gene sequences inserted to

the chromosomal DNA. The number of bands except for the endogenous *sip* signal in each transformant were as follows: Cp1-1, two bands; Cp1–2, two or three bands; Cp1–3, two bands; Cp1–4, one band; Cp1–5, three bands; Cp1–6, two bands. These results demonstrate that the introduced sequences (mutant sip) are integrated into the chromosomal DNA in these transformants with a copy number of one or more.

Hygromycin B resistance transformation

Four concentrations of hygromycin (150, 200, 300, and 400 µg/ml) were used in the regeneration plates for the hygromycin resistance transformants. More than half the colonies that appeared early in the experimental period did not grow into large colonies on the regeneration plates, and after transferring them to MYG plates containing 200 µg/ml hygromycin, they did not grow. The colonies that behaved in this way may not have had *hph* inserted in the genome and may have temporarily expressed resistance via rudimentary *hph* in the cell. Therefore, we counted the resistant transformants 1 week after the appearance of colonies. As the concentration of hygromycin B in the regeneration plates increased, the number of transformants that appeared decreased (see Table 2). No transformants were detected in the control (TE buffer) plates. The average transformation

Fig. 3. Southern hybridization analysis of carboxin-resistant transformants and the host strain. The position and size in kilobase pairs (kbp) are indicated on the *left*. *Bam*HI-digested DNA extracted from wild-type fungus (*lane 1*) and transformants (*lanes 2–7*) was probed with a portion of the sip sequence labeled with digoxigenin (*DIG*). *Arrow* indicates the wild-type band in the wild-type strain NGW19-6. *Lane 1*, NGW19-6; *lane 2*, Cp1–1; *lane 3*, Cp1–2; *lane 4*, Cp1–3; *lane 5*, Cp1–4; *lane 6*, Cp1–5; *lane 7*, Cp1–6

Table 2. Number of transformants/µg plasmid DNA for Hyg^r and Cbx^r transformation of *Pholiota microspora* strain NGW19-6

	Protoplasts number	Hygromycin B concentration (µg/ml)				Carboxin concentration (µg/ml)		
		150	200	300	400	0.5	1.0	2.0
Experiment 1	4.12×10^6	86.9	73.8	54.4	34.2	44.5	69.5	46.1
Experiment 2	4.45×10^6	153.4	133.6	60.6	41.2	95.8	125.3	79.0
Experiment 3	4.03×10^6	126.9	105.9	69.9	27.0	49.9	71.7	40.6
Average value		122.4	104.4	61.6	34.1	63.4	88.8	55.2

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efficiency for the plates with 150 μg/ml hygromycin was about 122.4 transformants/μg plasmid DNA using 4.5×10^6 viable protoplasts. In the regeneration plates containing 400 μg/ml hygromycin, the transformant colonies were sparse and small, suggesting that this concentration of hygromycin B is too high for *P. microspora*.

Compared with the carboxin-resistant colonies, the hygromycin B-resistant transformants grew more slowly on the regeneration plates and the MYG plates containing the relevant drug and had few aerial mycelia.

We used a host strain colony and seven transformants picked from the 150 μg/ml hygromycin B regeneration plates for PCR of a portion of *hph* with primers Hyg-F3 and Hyg-R3 (Fig. 4B) and Southern hybridization analysis (Fig. 4A). In these seven transformants, partial sequences of *hph* were obtained in all cases (Fig. 4A). For the host strain, no band appeared in either the PCR or the Southern hybridization analysis. The number of bands that appeared for each of the seven transformants in Southern hybridiza-

tion analysis were as follows: Hyg1–1, one band; Hyg1–2, two bands; Hyg1–3, one band; Hyg1–4, one band; Hyg1–5, two bands; Hyg1–6, two bands; Hyg1–7, one band. These results demonstrate that the introduced sequences (*hph*) are integrated into the chromosomal DNA in these transformants with a copy number of one or more.

In other mushroom species, such as *P. ostreatus* and *L. edodes*, for which transformation has been attempted transformation efficiency is very low when using normal polyethylene glycol-mediated transformation. Attempts have been made to improve transformation efficiency in *P. ostreatus* by using either chemical substances (heparin, ATA, and spermidine) (Li et al. 2006) or single-strand carrier DNA (Irie et al. 2001). However, in this study, we succeeded in establishing two highly efficient transformation systems in *P. microspora* using normal polyethylene glycol-mediated transformation, which produced a sufficient quantity of transformants for further molecular analysis of the cloned genes. This success may arise from features of the species

Fig. 4. Southern hybridization and polymerase chain reaction (PCR) analysis of hygromycin B-resistant transformants and the host strain. The position and size in kilobase pairs (kbp) are indicated on the *left*. **A** Southern hybridization analysis of hygromycin B-resistant transformants and the host strain. *Bam*HI-digested DNA extracted from wild-type fungus (*lane 1*) and transformants (*lanes 2–8*) was probed with a portion of the pMBhph1 sequence labeled with DIG. **B** PCR results of a portion of the hph gene in the host strain and hygromycin B-resistant transformants. *Lane 1*, NGW19-6; *lane 2*, Hy1–1; *lane 3*, Hy1–2; *lane 4*, Hy1–3; *lane 5*, Hy1–4; *lane 6*, Hy1–5; *lane 7*, Hy1–6; *lane 8*, Hy1–7
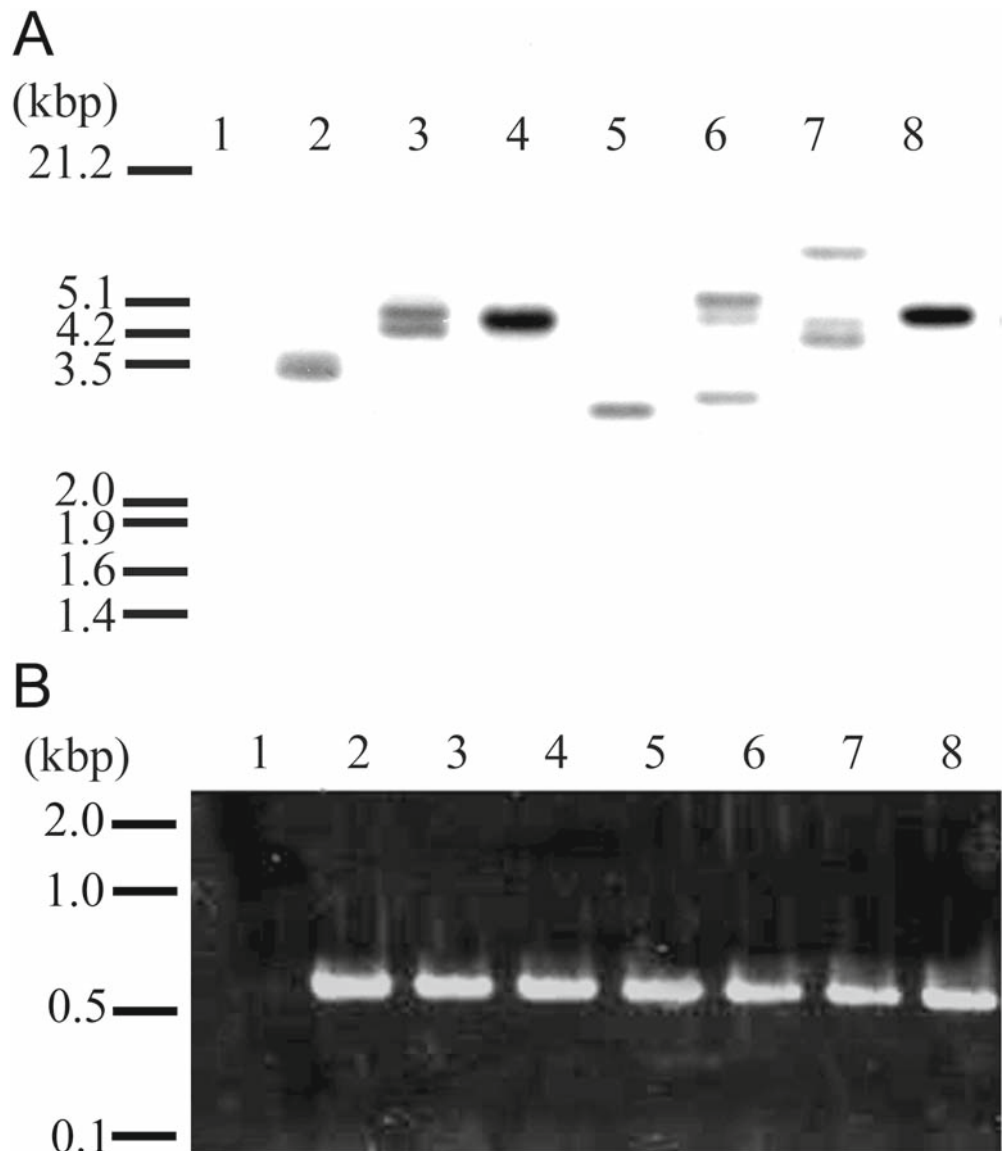
itself. Reports have shown that fungal protoplasts are very difficult to regenerate without any wall remnants (Bacon et al. 1969; Darling et al. 1969). It is possible that the protoplasts of *P. microspora* have more wall remnants than other mushroom species, which contributes to a high efficiency of regeneration. Unfortunately, we were unable to determine the regeneration efficiency of the *P. microspora* protoplasts because of mixing of the protoplasts with ungerminated oidia, which may germinate later.

In tetrapolar mushrooms, mating-type genes of *Coprinopsis cinerea* and *Schizophyllum commune* Fr. are well characterized (Raper 1966; Brown and Casselton 2001; Walser et al. 2001). In bipolar mushrooms, although cloning of mating-type loci has been reported in *P. microspora* (Aimi et al. 2005) and *Coprinellus disseminatus* (Pers.: Fries) J.E. Lange (James et al. 2006), it is not clear however that only the homeodomain protein can control mating compatibility and there is no experimental evidence about function of homeodomain proteins in the bipolar mushroom. In this study we successfully established construction of two selection markers for transformation. Normally, if a second gene is needed to introduce the transformed strain after the first transformation, a second selection marker is needed. Thus, in this research, we constructed two dominant selection markers in *P. microspora*. In the future, we will try to use these two transformation systems to investigate the functions of the homeodomain protein genes cloned in a previous study (Aimi et al. 2005).

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