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Cloning of glyceraldehyde-3-phosphate dehydrogenase gene and use of the *gpd* promoter for transformation in *Flammulina velutipes*

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Abstract The glyceraldehyde-3-phosphate dehydrogenase gene of *Flammulina velutipes* was isolated. The complete *gpd* sequence (from ATG to TAA) was 1,489 bp in length and contained nine introns. The locations of these nine introns were similar to those of other basidiomycetes, which might reflect the evolutionary divergence of these mushrooms. The *F. velutipes gpd* gene was found to encode a protein of 339 amino acids and its putative amino acid sequence revealed a high similarity to an analogous protein deriving from other basidiomycetes. Results of Southern blot analysis suggested that there existed only one copy of the *gpd* gene in the genome of *F. velutipes* and that there was one typical TATA box and two CAAT boxes located in the 5′ flanking region. The *F. velutipes gpd* promoter was fused to a hygromycin B phosphotransferase gene (*hph*) derived from *Escherichia coli* as a selection marker. Using the resulting construction, *hph* was efficiently transformed into *F. velutipes* by basidiospore electroporation. No false-positive antibiotic-resistant cultures were detected by PCR amplification and the hygromycin resistance trait was maintained stably during mitotic cell division for 3 months. Southern analysis of transformants indicated the integration of gene might occur by non-homologous recombination. This rapid and convenient electroporation procedure offers new prospects for the genetic manipulation and a tool for tagging genes of this important edible mushroom species.

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Sequence data will appear in the DDBJ/EMBL/GenBank nucleotide sequence database under accession number AF515622.

Introduction

Using strong promoters to express heterologous genes in appropriate hosts is a major strategy in biotechnological applications. The glyceraldehyde-3-phosphate dehydrogenase (GPD, EC 1.2.1.12) promoter is deemed a promising candidate. GPD is one of the key enzymes in the glycolytic and gluconeogenesis pathways and comprises up to 5% of the soluble cellular protein content in *Saccharomyces cerevisiae* and other higher eukaryotes (Piechaczyk et al. 1984). Furthermore, *gpd* mRNA accounts for 2–5% of the poly $(A)^+$ RNA present in yeasts (Holland and Holland 1978). These observations suggest that the *gpd* gene is regulated by a highly active promoter. In fact, vectors carrying the homologous *gpd* promoter region have been reported to be efficient in directing the expression of heterologous genes in yeasts (Bitter and Egan 1988; Doring et al. 1998; Eriksson et al. 1995; Vassileva et al. 2001) and filamentous fungi (Juge et al. 1998; Punt et al. 1987).

The *gpd* genes have also been cloned from basidiomycetous fungi, including *Schizophyllum commune*, *Phanerochaete chrysosporium*, *Agaricus bisporus* (Harmsen et al. 1992), and *Lentinula edodes* (Hirano et al. 1999). Among these mushrooms, genetic transformation using homologous *gpd* promoters was reported successful only in *A. bisporus* and *L. edodes* (Hirano et al. 2000; Van de Rhee et al. 1996). Although heterologous promoters have previously been used for the expression of drug-resistant marker genes, the genetic transformation was not sufficient to express heterologous genes (Ruiz-Diez 2002). To sufficiently and effectively express a heterologous gene, it is important for a host cell to recognize the promoter sequence by its transcriptional machinery.

In order to construct efficient transformation vectors allowing high-level expression of various heterologous

genes in mushrooms, it is necessary to isolate a highly active promoter and use this promoter to develop a genetic transformation system. Most protocols used in fungal transformation involved electroporation of protoplasts (Chakraborty et al. 1991; Robinson and Sharon 1999; Van de Rhee et al. 1996; Ward et al. 1989), treatment with $CaCl_2$ and polyethylene glycol (Ogawa et al. 1998; Sato et al. 1998), or restriction enzyme-mediated integration (Hirano et al. 2000; Irie et al. 2003; Sato et al. 1998). Since these transformation systems mainly relied on troublesome protoplast preparation, they were not easy to apply to all edible mushrooms, yielding insufficient regenerable protoplasts. *Agrobacterium tumefaciens*-mediated transformation is routinely used for the genetic modification of a wide range of plant species and demonstrates the ability to transfer DNA from a prokaryote to filamentous fungi (Chen et al. 2000; Combier et al. 2003; De Groot et al. 1998; Dunn-Coleman and Wang 1998; Mikosch et al. 2001). However, this method is not necessarily appropriate for all mushroom species.

In this study, we cloned the *gpd* gene from *Flammulina velutipes*, one of the most important edible mushrooms in Asia, and analyzed its complete nucleotide sequence, including the 5′ flanking region. We also developed a simple and reliable electroporation protocol for basidiospores. Using this protocol and the homologous *gpd* promoter, we demonstrated that a heterologous gene, the hygromycin B phosphotransferase gene (*hph*), can be successfully expressed in *F. velutipes* and this protocol can be applied to most mushroom species without special equipment or techniques.

Materials and methods

Strains and media

F. velutipes BCRC 37086 was purchased from the Bioresources Collection and Research Center (Hsinchu, Taiwan) and grown in either potato dextrose agar (PDA; Difco, Detroit, Mich.) or potato dextrose broth (PDB;

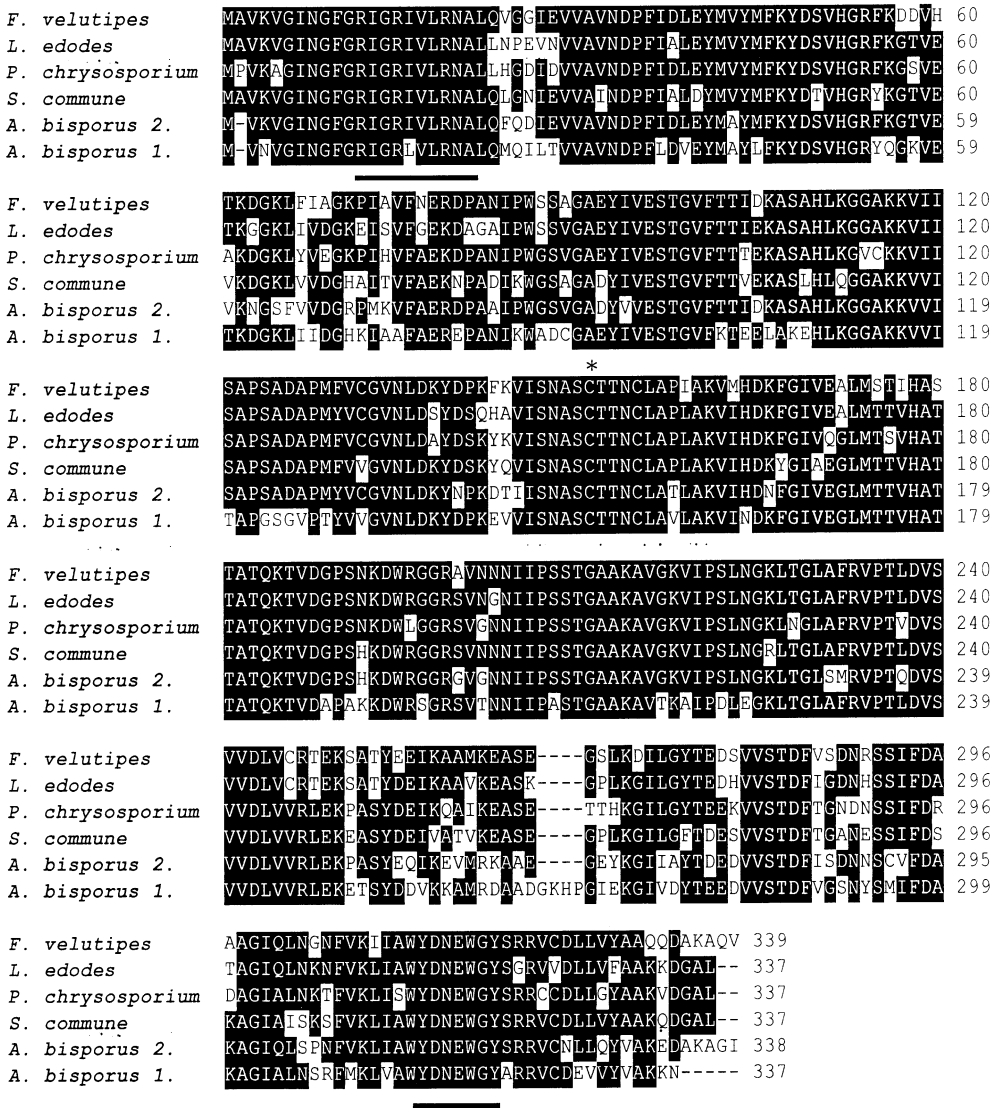

Fig. 1 Alignment of the deduced amino acid sequences of the GPD proteins of *F. velutipes*, *L. edodes*, *Agaricus bisporus*, *P. chrysosporium*, and *S. commune*. Identical residues are *highlighted*. Two conserved regions for degenerate oligonucleotide primers design are *underlined*. The main catalytic amino acid residue cysteine at position 151 is indicated by an *asterisk*

Difco) at 25°C. Transformants were selected on PDA with 30 μg/ml of hygromycin (A.G. Scientific, San Diego, Calif.). *Escherichia coli* strain DH5α (GIBCO–BRL Life Technologies, Grand Island, N.Y.) was used for DNA manipulations and grown in LB medium (Sigma Chemical Co., St Louis, Mo.) at 37°C. PCR-amplified DNA fragments were purified, cloned into a pGEM-T Easy vector (Promega, Madison, Wis.), and sequenced.

Cloning of the *gpd* gene

Two degenerate primers, 5′-GKATCGGMCGYMYYGT-MYYCMGHAATGC-3′ (corresponding to RIGRIVLR-NA) and 5′-RTANCCCCAYTCRTTRTCRTACCA-3′ (corresponding to WYDNEWGY), were designed according to the conserved amino acid sequences of published basidiomycetous *gpd* genes (GenBank accession nos. BAA83550, AAA33926, AAA33732, AAA32634, AAA32633; Fig. 1). Using these degenerate primers and *F. velutipes* genomic DNA as the template, partial *gpd* of *F. velutipes* was cloned by PCR amplification.

The 5′ flanking region and *cDNA* of *F. velutipes gpd* was cloned by means of the Clontech Universal GenomeWalker kit and SMART RACE cDNA amplification kit, according to the manufacturer's instructions, respectively (BD Bioscience, Paolo Alto, Calif.). A gene-specific primer (GSP) was designed from the partial *F. velutipes gpd* sequence.

Plasmid construction

The hygromycin B phosphotransferase (*hph*) gene with cauliflower mosaic virus (CaMV) 35S terminator was amplified from pCAMBIA 1300 (CAMBIA, Canberra, Australia) using primers Hyg-f (5′-ACTAGTAT-GAAAAAGCCTGAACTCACC-3′) and Hyg-r (5′-CTGCAGACAACTTAATAACACATTGCG-3′). Then, the amplified fragment was cloned into a pGEM-T Easy vector and a 1.4-kb promoter region of the *F. velutipes gpd* gene was introduced to drive *hph* gene expression. The resulting plasmid, pFGH, was used for the transformation experiments.

Transformation procedure

Exponential-decay high-voltage electric pluses were delivered by BTX ECM 630 and 0.2-cm cuvettes (BTX, San Diego, Calif.). The electric pulse delivery test used several settings: capacitor 25 μF, resistance from 100 Ω to 800 Ω, and field strength from 6.25 kV/cm to 12.5 kV/cm.

Basidiospores were collected from *F. velutipes* fruit bodies, suspended in PDB, and then incubated overnight with gentle shaking at 25°C. These germinated basidiospores were harvested by centrifugation at 2,000 *g* for 5 min and resuspended in P buffer (0.02 M phosphate buffer, pH 5.8, 0.6 M mannitol) containing 2 mg/ml lysing enzymes (Sigma). After incubation for 2 h, these basidiospores were washed free of enzyme and transferred to a small volume of electroporation buffer (1 mM Hepes, pH 7.5, 0.6 M mannitol). Basidiospores (10^7–10^8) were mixed with 10 μg plasmid DNA, chilled on ice for 10 min, and subjected to electroporation. After pulse delivery, basidiospores were kept for 10 min on ice and mixed with PDB containing 0.6 M mannitol. Transformants were selected on PDA plates containing 30 μg/ml hygromycin.

Detection of introduced sequence in transformants and stability test

Genomic DNA isolated from putative hygromycin-resistant transformants was analyzed by PCR. Amplification of *hph* gene was carried out using primers Hyg-f and Hyg-r, previously used in plasmid construction. The amplified fragments were further identified by restriction enzyme *Xho* I digestion. Randomly selected transformants were transferred to medium without antibiotic selection for weeks to months, followed by a hygromycin resistance test.

Southern hybridization

Approximately 5 μg of genomic DNA digested with restriction enzymes were size-fractionated by electrophoresis on a 1% agarose gel; and the DNA fragments in the agarose gel were transferred to a Hybond N^+ Nylon membrane (Amersham, Hong Kong) using 10× SSC. A genomic DNA fragment amplified by PCR with two *gpd* gene-specific primers (5′-GATCGGCCGTCTTGTCCTCC-3′, 5′-GCAATTGG-TAGTGCAAGAAGCG-3′) was used as a subsequent probe for *gpd*. The DNA fragment amplified by PCR with primers Hyg-f and Hyg-r from pCAMBIA 1300 was used as a subsequent probe for transformants. Labeling of the DNA probe, hybridization, and signal detection were conducted by means of the digoxigenin (DIG)-probe synthesis and detection kit (Roche, Mannheim, Germany), according to the manufacturer's instructions.

Results

Isolation and structure of *F. velutipes gpd*

Using degenerate primers designed according to conserved amino acid sequences of published basidiomycetous *gpd* genes, a fragment approximately 1.2 kb in size was amplified by PCR from the *F. velutipes* genomic DNA template. The 5′ flanking region of the *F. velutipesgpd* gene was amplified by genome walking and the full-length *gpd* cDNA of *F. velutipes* was obtained by aligning sequences obtained by 5′RACE and 3′RACE.

Based upon the alignment of cDNA sequences and a comparison with the genomic DNA sequence, the

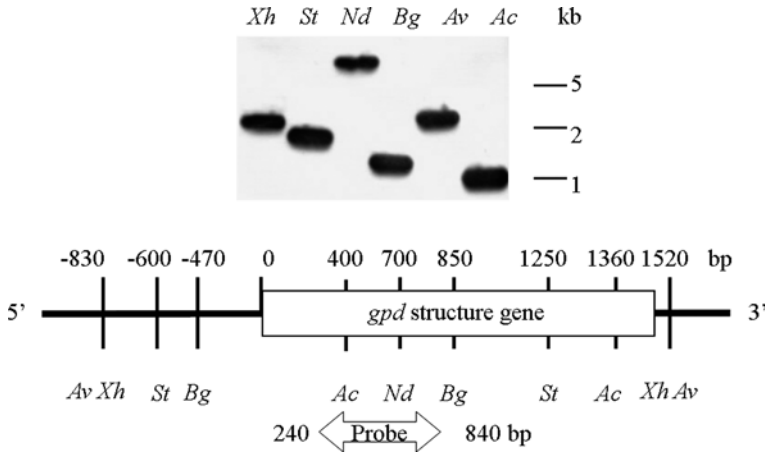

Fig. 2 Southern blot analysis of genomic DNA and an illustration of the *gpd* gene. *Numbers* indicate the positions of restriction enzyme cutting sites for the *gpd* gene. A 600-bp genomic DNA fragment amplified by PCR using two *gpd* gene-specific primers was used as a probe. *Xh Xho* I, *St Stu* I, *Nd Nde* I, *Bg Bgl* II, *Av Ava* I, *Ac Acc* I

complete *gpd* sequence (from ATG to TAA) of *F. velutipes* is 1,489 bp in length, with the presence of nine introns (DDBJ/EMBL/GenBank nucleotide sequence database, accession number AF515622). The positions of introns were highly conserved in the *gpd* genes of the investigated basidiomycetes (Harmsen et al. 1992), especially *A. bisporus* (data not shown), which might reflect the evolutionary divergence of these mushrooms.

The GPD protein of *F. velutipes* is encoded by 339-amino acids, and the amino acid sequence is similar to that of other species (Fig. 1). The main catalytic amino acid residue responsible for the binding of glyceraldehyde-3-phosphate within the *F. velutipes* GPD protein is found at the 151st residue, cysteine. This observation was also found in other basidiomycetes including *L. edodes*, *A. bisporus*, *P. chrysosporium*, and *S. commune* (Hirano et al. 1999).

The sequence of the 5′ flanking region

There was one typical TATA box (TATAAAA) and two CAAT boxes (CCAAT) located in the 5′ flanking region. The CAAT boxes were located further upstream from the initiating ATG codon than in other published basidiomycetous *gpd* genes (Harmsen et al. 1992; Hirano et al. 1999). A pyrimidine region composed of stretches of thymine nucleotides interrupted by cytosine residues was observed immediately upstream of the transcription initiation site. Similar to other basidiomycetes (Harmsen et al. 1992), those consensus-promoter elements labeled as the *gpd* box, *pgk* box, *qut* box, and *qa* box in the *A. nidulans* and *A. niger gpd* promoter regions (Punt et al. 1990) were not found in the promoter region of the *F. velutipes gpd* gene.

Copy number of the *gpd* gene

Southern hybridization was conducted in order to detect the copy number of the *gpd* gene in the *F. velutipes* genome. Digested *F. velutipes* genomic DNA fragments were hybridized with a DIG-labeled genomic DNA fragment amplified by PCR using two *gpd* gene-specific primers. The expected specific *gpd* signals were detected following Southern hybridization (Fig. 2), suggesting that there is only one copy of *gpd* gene in the genome of *F. velutipes*. A similar result was also found in *L. edodes* (Hirano et al. 1999).

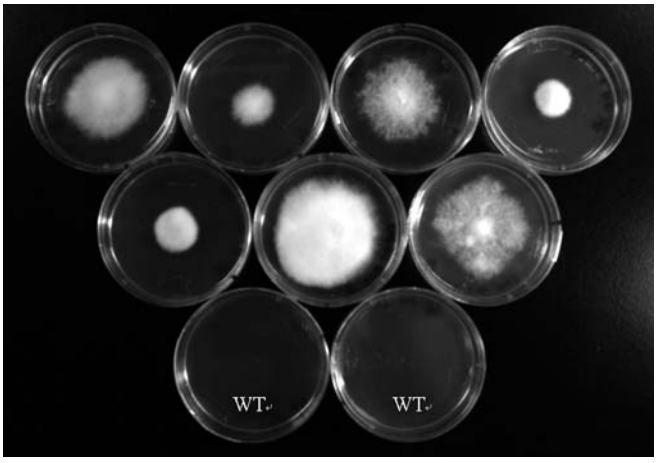

Fig. 3 Transformants expressed the hygromycin resistance trait stably under a non-selective environment. Randomly selected transformants were subcultured on medium without hygromycin for 3 months and then transferred to PDA plates containing 30 μg/ml of hygromycin. *WT* Wild-type non-transformed parental strain

Transformation of *F. velutipes* basidiospores

Basidiospores after transformation were colonized on PDA with and without 30 μg/ml of hygromycin, respectively. The transformation efficiencies ranged over 5–60 transformants/μg DNA at all electric pulse delivery settings. This was in the same rank as results obtained by the REMI method (Hirano et al. 2000), but avoided protoplast preparation.

Subculturing transformants on media without selection pressure and followed by a hygromycin resistance test demonstrated that the hygromycin resistance trait was maintained stably during mitotic cell division for 3 months (Fig. 3). The *gpd* promoter of *F. velutipes* constitutively

drove the hygromycin resistance trait, indicating the promise of downstream gene expression. The presence of *hph* DNA introduced via transformation was checked by PCR amplification and identified by *Xho* I digestion of the PCR-amplified fragments (Fig. 4). No false positives were detected by PCR amplification among 30 antibiotic-resistant cultures. In order to investigate the fate of transforming DNA, Southern blot analysis was performed for transformants (Fig. 5). Genomic DNA from transformants was digested with *Eco*R I and hybridized with a DIG-labeled *hph* gene probe. There is only one *Eco*R I cutting site within the plasmid pFGH and therefore hybridization bands could be used to determine the copy number of *hph* per genome. Figure 5 shows that the *hph* gene integrated one or three copies in tested transformants; and bands of various sizes were visualized. This result suggested that the introduced fragment was integrated randomly into the *F. velutipes* genome.

Discussion

F. velutipes gpd

By comparison of the DNA and cDNA sequences obtained above, the coding region of genomic *gpd* DNA was determined. It agreed perfectly with the cDNA clone, suggesting this *gpd* gene encodes a functional protein. Some fungi, such as *Mucor circinelloides* and *A. bisporus*, which harbor more than one *gpd* gene, contained only one *gpd* mRNA, indicating that there is only one functional *gpd* (Harmsen et al. 1992; Wolff and Arnau 2002). It is thus plausible to assume that the *gpd* isolated from *F. velutipes* should be a functional gene. The result of Southern analysis suggested that there is only one copy of the *gpd* gene in the genome of *F. velutipes*. In the case of *Nde* I and *Acc* I, there should be two positive bands rather than one. This might be due to incomplete digestion and non-optimal hybridization stringency. The ability of the promoter obtained in this study to drive heterologous gene expression was proved by the hygromycin resistance trait of transformants; and this indicated the promise of other downstream gene expression. To determine which part of the *gpd* promoter region is essential for its function, deletion analysis of shortened *gpd* promoter will start in the future.

Transformation of *F. velutipes* basidiospores

The results indicated that electroporation of basidiospores could be a useful method for basidiomycete transformation and that the *gpd* promoter of *F. velutipes* was useful in the expression of foreign genes. Our results also showed that the cell wall acts as a powerful barrier to the uptake of DNA during electroporation; and this is consistent with observations in other filamentous fungi (Chakraborty et al. 1991). The mild pretreatment of germinated basidiospores with lysing enzymes proved useful, since it did not

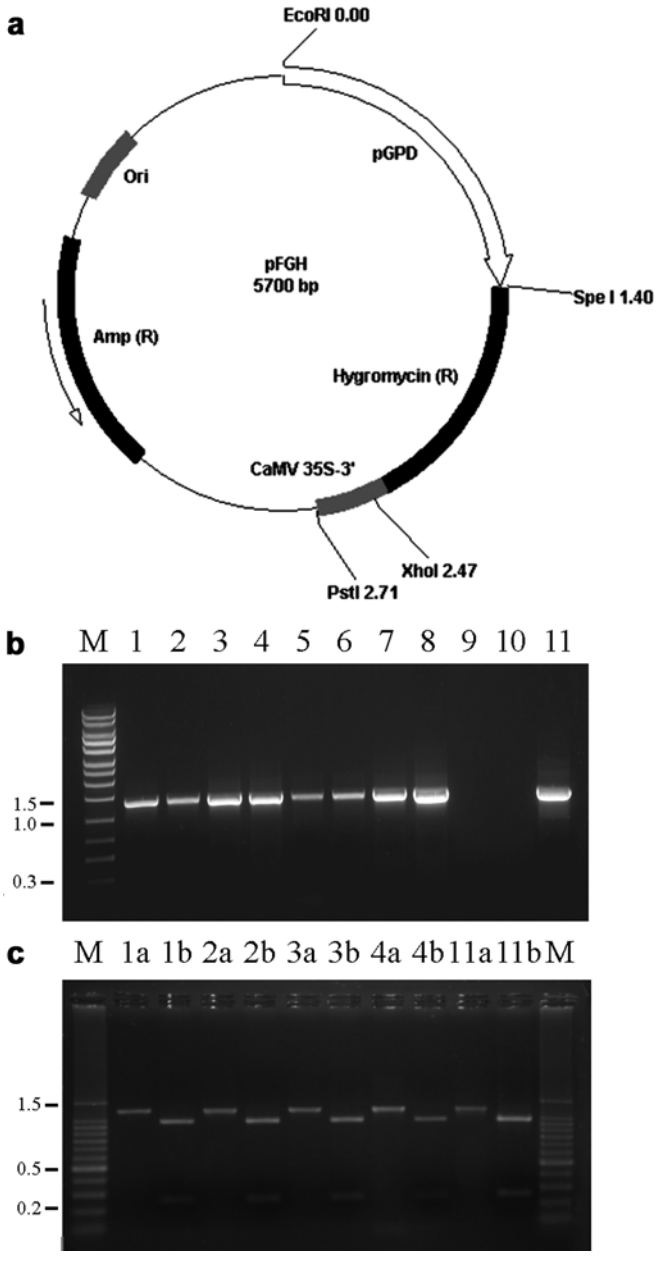

Fig. 4 **a** Organization of pFGH. pFGH is 5.7 kb in size and consists of a pGEM-T Easy vector backbone containing the ampicillin resistance gene [*Amp (R)*]. The hygromycin resistance gene [*Hygromycin (R)*], with the CaMV35S terminator (*CaMV 35S-3'*), was joined to the *F. velutipes gpd* promoter (*pGPD*). Shown are restriction enzyme sites with map distance in kilobases. **b** PCR analysis of DNA isolated from putative hygromycin-resistant transformants. PCR amplification was carried out on genomic DNA using primers Hyg-f and Hyg-r defining a ca. 1.3-kb fragment containing the *hph* gene and CaMV 35S terminator. *Lanes 1–8* DNA from randomly selected putative transformants, *lane 9* negative control with water, *lane 10* DNA isolated from non-transformed *F. velutipes*, *lane 11* positive control with plasmid pFGH. **c** Identification of PCR-amplified fragments from **b**. *Lanes 1–4* amplified fragments from lanes 1–4 of **b**, *Lane 11*, positive control with plasmid pFGH from lane 11 from **b**. *a, b* Respectively, treated without or with restriction enzyme *Xho*I, *M* DNA molecular size markers (kilobases)

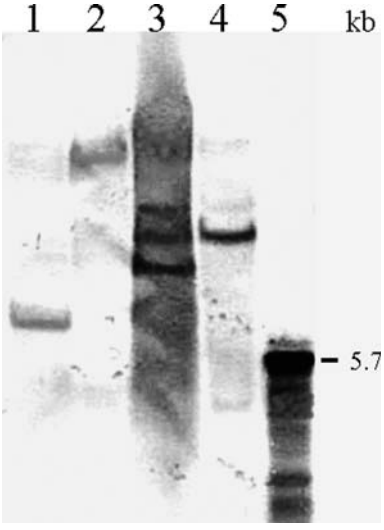

Fig. 5 Southern blot analysis of transformants. *Lanes 1–4 Eco*RI-digested genomic DNA of transformants probed with the DIG-labeled *hph* sequence, *lane 5* positive control of pFGH

seriously compromise the integrity of the cell wall or the viability of basidiospores, while it elicited a marked enhancement in the yield of transformants.

The use of basidiospores instead of vegetative mycelium as recipient provided an easy way to determine transformants without the false positives caused by tissue tolerance toward antibiotics and made the electroporation procedure more applicable. The variation in the copy number and position of *hph* between transformants indicated the integration of gene was a random event and might occur by non-homologous recombination (Fig. 5). No clear relationship between the diversity of integrated DNA and morphological differences nor the *hph* copy number and growth rate was found in this study. This came as no surprise, since each monokaryon germinated from a basidiospore has a different genomic background, consequently leading to a different morphology (Brown and Casselton 2001; Larraya et al. 2001). One additional advantage of using basidiospores in transformation is the ease of tracing heterologous genes, since the transformants are monokaryons instead of heterokaryons.

Although *Agrobacterium tumefaciens*-mediated transformation is useful in plants and some filamentous fungi, it does not work with *F. velutipes*, suggesting the species hedge might limit its extensive application. In addition, mushrooms are heterokaryons and it is easier to trace heterologous genes in transformants produced by basidiospore electroporation. The transformation procedure used in this study could be used in *F. velutipes* and other edible mushrooms, such as *Agaricus bisporus* and *L. edodes* without troublesome protoplast preparation, cocultivation, or expensive equipment. This procedure provides a tool for mushroom genetic research and makes molecular breeding a reality. This is the first report about transformation and expression of heterologous genes in the important edible mushroom *F. velutipes*.

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