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A reliable transformation method and heterologous expression of β -glucuronidase in *Lentinula edodes*

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Abstract

A simple and reliable mushroom transformation procedure based on electroporation of basidiospores or mycelial fragments was developed. This method eliminated the problem of protoplast preparation, the transformation efficiency were 30-150 transformants per µg DNA and the hygromycin resistant marker gene and *gus* were expressed in *Lentinula edodes* successfully. No false positive antibiotic-resistant cultures were detected by PCR amplification and the β -glucuronidase (GUS) expression was maintained stable during mitotic cell division without selection pressure for more than 6 months. Southern analysis of transformants indicated the integration of gene might occur by non-homologous recombination. Using the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter with the first intron of *gpd* gene, the average GUS activity in *L. edodes* reached 144.6± 3.9 U mg⁻¹ soluble protein, while only 30.1 ± 0.7 U mg⁻¹ soluble protein was detected for those without the intron. The percentage of GUS in total soluble protein was 5.67×10^{-4} (0.06%) for the transformant with the highest GUS activity. This rapid and convenient electroporation procedure offers a new approach for the genetic manipulation and tool to tag genes of important edible mushroom species. © 2007 Elsevier B.V. All rights reserved.

Keywords: Electroporation; β-glucuronidase; Heterologous expression; Lentinula edodes

1. Introduction

Lentinula edodes, or shiitake, is the most important edible basidiomycetes in Asia. In consideration of molecular breeding and the potential of using mushrooms as expression hosts, researchers have put many efforts in development of appropriate genetic transformation systems for edible mushrooms. Though genetic transformation systems using auxotrophic and drug-resistant markers have been developed for many basidiomycete fungi (Chen et al., 2000; Hirano et al., 2000; Peng et al., 1992; Ruiz-Diez, 2002), an efficient and reliable transformation and heterologous expression system for mushrooms still requires further development.

In the past, most protocols used in fungal transformation involved electroporation of protoplasts (Chakraborty et al., 1991; Robinson and Sharon, 1999; van de Rhee et al., 1996),

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treatment of CaCl₂, 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). Only a few reports demonstrated the transformation of L. edodes (Hirano et al., 2000; Irie et al., 2003; Li et al., 2006; Sato et al., 1998). Since these transformation systems mainly relied on troublesome protoplast preparation, it was not applicable to other edible mushrooms which may not yield sufficient regenerable protoplasts and these transformation events might be inefficient or difficult to reproduce in other laboratories. Agrobacterium tumefaciens-mediated transformation has been routinely used for the genetic modification of a wide range of plant species and also demonstrated the ability to transfer DNA from a prokaryote to filamentous fungi (Chen et al., 2000; Combier et al., 2003; De Groot et al., 1998; Leclerque et al., 2004; Mikosch et al., 2001), nevertheless, this method is not necessarily appropriate to all mushroom species.

In our previous study, a transformation procedure based on basidiospore electroporation for *Flammulina velutipes* had been developed (Kuo et al., 2004). In this study, we demonstrated

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Table 1

Primers used in plasmids construction	
Target genes	Primers used
I edodes nand	pL-f (5' CCATCCTCGATAT

pL-f (5' CCATGG TCGATATCAGTCAGATTG 3')
pL-r (5' ACTAGTGGCCATTTTAATTCAAGC 3')
pL-ir (5' ACTAGTGGCCTGAAAAGACATGG 3')
Gus-f (5' ACTAGTTTACGTCCTGTAGAAACC 3')
Gus-r (5' GAGCTCGTTTTCAGAAGACGGCTG 3')

Restriction enzyme cutting site were shown in bold script.

that the high transformation efficiency and hetelogous expression of *gus* in *L. edodes* could be achieved by a simple and reliable electroporation procedure for mycelial cells transformation. This procedure eliminated the problem of protoplast regeneration and the limitation of host specificity and could be a useful method for mushroom transformation.

2. Materials and methods

2.1. Strains and media

L. edodes strain LD 106 was acquired from the culture collection of the Laboratory of Applied Microbiology, Institute of Microbiology and Biochemistry, National Taiwan University. Basidiomycetes were grown in either PDA (Potato dextrose agar, Difco, Detroit, MI, USA) or PDB (Potato dextrose broth, Difco) at 25 °C. Transformants were selected on PDA with 30 μ g ml⁻¹ Hygromycin (Sigma, St. Louis, MO, USA). The *Escherichia coli* DH5 α (GIBCOBRL, Life Technologies, Grand Island, NY, USA) was used for DNA manipulations and grown in LB medium (Sigma) at 37 °C.

2.2. Plasmid construction

Two plasmids pL-gus and pLi-gus were constructed based on the backbone of plasmid pFGH (Kuo et al., 2004). Primers



Fig. 1. Organization of pL-gus/pLi-gus for the expression of *gus* as reporter gene. The hygromycin-resistance gene (*hpt*) was joined to the *gpd* promoter (*pgpd*), *gus* was driven with *pgpd* or *pgpdi* to produce pL-gus or pLi-gus. NOS poly A: nopaline synthase poly A signal. CaMV35 S poly A: CaMV35 S (cauliflower mosaic virus 35 S) poly A signal. Amp: the ampicillin resistance gene. The arrowheads indicates the positions of the primers.



Fig. 2. PCR analysis of DNA isolated from putative transformants. PCR amplification was carried out on genomic DNA using primers Gus-f and Gus-r defining a ~ 2.0 kb fragment containing *gus* gene and nopaline synthase poly A signal. Lanes: 1 to 8, DNA from randomly selected putative transformants; N, negative control with DNA isolated from nontransformed *L. edodes*; P, positive control with plasmid transformed.

used to amplify promoter regions (*pgpd*) and reporter gene *gus*-Nos poly A are listed in Table 1. Reverse primer pL-ir and pL-r was used to amplified *pgpd* from *L. edodes* genomic DNA with (*pgpdi*) or without (*pgpd*) the first intron of *gpd* gene, respectively, while *gus*-Nos poly A was amplified from pCAMBIA 1391 (CAMBIA, Canberra, Australia). Hygromycin resistant gene (*hpt*) was under control of *pgpd* without intron, but *gus* was driven with *pgpd* or *pgpdi* to produce pL-gus or pLi-gus. The schematic composition of the resultant plasmids used for the transformation experiments is illustrated in Fig. 1.

2.3. Transformation procedure

Exponential-decay high voltage electric pluses were delivered by BTX ECM 630 and 0.2-cm cuvettes (BTX, San Diego, CA). The electric pulse delivery test conditions include several settings: capacitor 25 μ F; resistor from 100 Ω to 800 Ω and field strength from 6.25 kV cm⁻¹ to 12.5 kV cm⁻¹.

Basidiospores were collected from *L. edodes* fruiting bodies, suspended in PDB and incubated overnight with gentle shaking at 25 °C. These germinated basidiospores were harvested by centrifugation at 2000 g for 5 min and resuspended in P buffer (0.02 mol l^{-1} phosphate buffer, pH 5.8, 0.6 mol l^{-1} mannitol)



Fig. 3. Southern-blot analysis of the transformants. Lane 2-7, 9-14: Spe I-digested genomic DNA of transformants was probed with the DIG-labeled *gus* sequence. Lane 8: negative control of wild type genomic DNA. Lane 1: DNA molecular size markers (kilobases). A 2.0 kb PCR product of *gus* gene and the linearized pL-gus of 6.4 kb, were used as the molecular size marker.



Fig. 4. Histochemical detection of GUS in *L. edodes*. Transformants were cultured in 10 mL PDB. After 10 days of incubation, the mycelia were washed in detection buffer and incubated at 37 °C for 3 h in detection buffer containing 0.5 mg/mL X-gluc. (A), (C): transformants; (B) wild type. (A), (B): 40X, Bar=100 µm; (C): 400X, Bar=10 µm.

containing 2 mg ml⁻¹ lysing enzymes (Sigma). After incubation for 2 h, these basidiospores were washed free of enzyme by centrifugation and resuspended in electroporation buffer (1 mmol 1^{-1} HEPES, pH 7.5, 0.6 mol 1^{-1} mannitol) twice. About $10^7 - 10^8$ basidiospores suspended in 100 µl electroporation buffer were mixed with 10 µg plasmid DNA, chilled on ice for 10 min, and subjected to electroporation. After pulse delivery, basidiospores were kept on ice for 10 min and mixed with 900 μ l PDB containing 0.6 mol 1⁻¹ mannitol. Transformants were selected on PDA plates containing 30 $\mu g m l^{-1}$ hygromycin. The mycelium-based transformation procedure modified from above technique was also developed. Four-dayold liquid cultures of L. edodes mycelia were blended with Waring blender, and then incubated overnight with gentle shaking at 25 °C. Mycelial fragments were collected by centrifugation at 3000 g and washed with P buffer. After treating with lysing enzyme as described above, $c. 100 \ \mu l$ mycelial pellets were resuspended by 200 µl electroporation buffer and mixed with 10 µg plasmid DNA. The electroporation conditions described above were applied.

2.4. Detection and stability of the introduced sequence in the transformants

Genomic DNA isolated from putative hygromycin-resistant transformants was analyzed by PCR. Amplification of *gus* gene was carried out using primers Gus-f and Gus-r previously used in plasmid construction. The *hpt* gene stability was assayed by transferring randomly selected transformants to medium without antibiotic selection for weeks to months, and followed by hygromycin-resistant test.

2.5. Southern hybridization

Approximately 5 µg of genomic DNA digested with restriction enzymes was size-fractionated by electrophoresis on a 1% agarose gel. The DNA fragments in the agarose gel were transferred to a Hybond N⁺ nylon membrane (Amersham, Hong Kong) using $10 \times SSC$. The DNA fragments containing *gus* amplified by PCR from pCAMBIA 1391 was used as a probe for southern hybridization. Labeling of the DNA probe,



Fig. 5. Determination of GUS activities in transformants. GUS activities were present as unit per mg soluble protein extracted from mycelium. Top ten of transformants from both constructions were explained, error bars indicate standard deviations of three independent experiments.

hybridization, and signal detection were conducted by means of the Roche DIG-probe synthesis and detection kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

2.6. Detection of GUS in transformants

For detection of B-glucuronidase activity, transformants were cultured in 10 ml PDB. After 10 days of incubation, the mycelia were washed with detection buffer (0.5% Triton X-100, 0.1 mol l^{-1} sodium phosphate, pH 7.0) and incubated at 37 °C for 3 h in detection buffer containing 0.5 mg ml⁻¹ X-gluc (5-Bromo-4chloro-3-indolyl-B-D-Glucuronic Acid, Cycolhexylammonium Salt, Sigma). Microscopic observation was performed by an Olympus BH-2 Microscope (Tokyo, Japan). Assay for GUS activity were done by B-glucuronidase fluorescent reporter gene activity detection kit (Sigma). Mycelium was frozen in liquid nitrogen and ground with a pestle. Protein extraction and activity determination were conducted according to the manufacturer's instructions. The fluorescence intensity of 4-MU was measured by the fluorometer FluoroMax-3 (Jobin Yvon and Glen Spectra, Edison, NJ). One unit is defined as the amount of enzyme that release one pmole of 4-MU from 4-MUG per minute at pH 7.0 and 37 °C. Protein concentrations were determined by using a bicinchoninic acid assay (Pierce, Dallas, TX). The GUS expressed in E. coli by pET21a(+) was served as a positive control enzyme.

3. Results

3.1. Transformation of L. edodes

According to the procedures developed in our previous study (Kuo et al., 2004), the transformation efficiency using germinated basidiospores of *L. edodes* or *F. velutipes* was about 50 transformants per μ g DNA. Genetic transformation could be achieved

in all electroporation settings, higher resistor and field strength might increase the transformation efficiency, however, the chance of cuvette explosion increased too. The routine setting was capacitor 25 μ F; resistor 200 Ω and field strength 10 kV cm⁻¹. In contrast, the transformation efficiency using small mycelial fragments was about 30 transformants per μ g DNA. The growth rate and morphology showed no significant difference between transformants and the wild type strain (data not shown).

Subculturing transformants on media without selection pressure and followed by hygromycin-resistant test demonstrated that hygromycin resistance trait remained stable during mitotic cell division for at least 6 months. Fig. 2 shows the presence of *gus* DNA introduced via transformation checked by PCR amplification. No false positives were detected among 50 antibiotic-resistant cultures. In order to investigate the fate of transforming DNA, Southern hybridization was performed and the results are shown in Fig. 3. Southern hybridization analysis confirmed that multicopy integration of the heterologous genes occurred in the chromosomal DNA of tested transformants as evidenced by bands of various sizes. This result also suggested that introduced fragment was integrated randomly into the genome.

3.2. Detection of GUS in transformants

For histochemical detection of GUS in *L. edodes*, the colonies that appeared on selective medium were cultured in 10 ml PDB. After 10 days of incubation, the mycelia were collected for GUS detection. GUS activity was identified by formation of blue product from X-gluc (Fig. 4 A, C), while the wild type host strain did not show blue color (Fig. 4 B). Only 40–60% of *L. edodes* transformants showed GUS activity, though either *pgpd* or *pgpdi* could be used to drive *gus* gene expression. GUS expression remained stable through multiple rounds of subculturing without selection pressure for more than 6 months.

3.3. Activity determination and expression level of GUS in *L. edodes*

Transformants confirmed by histochemical detection were chosen for GUS activity assay. Mycelia of thirty randomly selected transformants for *gus* driven by *pgpd* or *pgpdi* construction were washed free of medium, frozen and ground into fine powder, and followed by protein extraction. Fig. 5 shows the GUS activities of the top ten transformants from each construction. When *gus* driven by *pgpdi*, containing the first intron of *gpd* gene, the average activity was $144.6\pm3.9 \text{ U mg}^{-1}$. In contrast, there were only $30.1\pm0.7 \text{ U mg}^{-1}$ in average for the transformants without the intron.

The purified GUS expressed from *E. coli* was used to figure out the specific activity. The specific activity of purified GUS was 4.94×10^5 U mg⁻¹, i.e., 1 U of GUS activity was equivalent to 2 ng GUS protein. The highest GUS activity among the transformants was 283.3 ± 4.91 U mg⁻¹ soluble protein, indicating that there was 566 ng GUS protein per mg soluble protein and the expression level was $5.66 \times 10^{-4} (0.06\%)$.

4. Discussion

4.1. Transformation of L. edodes

In the past, the conventional PEG transformation of *L. edodes* yielded only 1.5 transformants per 25 μ g plasmid DNA (Sato et al., 1998). Although restriction enzyme-mediated integration (REMI) could increase the efficiency 100 fold to 16 transformant per μ g plasmid DNA (Hirano et al., 2000; Sato et al., 1998), these transformation systems still mainly relied on troublesome protoplast preparation. In this study, a simple and reproducible procedure based on germinated basidiospores or mycelia electroporation successfully transformed *L. edodes* with higher efficiency and the heterologous *gus* gene expression. The mild pretreatment of germinated basidiospores and mycelia fragments with lysing enzymes proved useful since it did not seriously compromise the integrity of the cell wall or the viability, while eliciting a marked enhancement in the yield of transformants.

The variation of the copy number and location of heterologous genes between transformants indicated the integration of gene was a random event and might occur by non-homologous recombination (Fig. 3). No clear relationship between the diversity of integrated DNA and heterologous gene expression was found in this study. In our experience, the more gene cassettes in a vector lead to the less transformation efficiency. Using the same construction without the reporter gene expression cassette, the transformation efficiency was more than 150 hygromycin resistant transformants per μ g DNA, in comparison to 50 transformants per μ g DNA using a plasmid containing both *gus* and *hpt* genes. This might be attributed to the different size of final constructions, and smaller DNA fragment might be easier to integrate into chromosome. This transformation efficiency in *L. edodes* was higher than other report (Li et al., 2006).

4.2. Expression of GUS in L. edodes

There are only a few paper reporting the transformation of L. edodes (Hirano et al., 2000; Irie et al., 2003; Li et al., 2006; Sato et al., 1998), and none of them demonstrated the heterologous gene expression. In this study, we demonstrated that the heterologous gus gene could be expressed by gpd promoter with or without the first intron of gpd gene. When driven by gpd promoter with the intron, the average activity was $144.6\pm$ 3.9 U mg^{-1} soluble protein, almost 5 folds of the construction without the intron $(30.1 \pm 0.7 \text{ U mg}^{-1})$. The enhancement of gene expression by presence of intron were also seen in plant including Arabidopsis (Norris et al., 1993) and rice (Tanaka et al., 1990). The enhancement of foreign gene expression might be correlated with an increased level of mRNA and an efficient splicing of the intron (Tanaka et al., 1990). However, the role of the first intron of gpd in driving gus gene in L. edodes still needs further investigation.

The results demonstrated that the electroporation procedure used in this study offers an efficient method for mushroom transformation without the troubling protoplast preparation. Since it does not require protoplast isolation and regeneration, the procedure is simple, reliable and reproducible. This protocol could be employed not only for *L. edodes*, but also for other edible mushrooms such as *F. velutipes* and *Pleurotus ostreatus* (unpublished data). We believe that this method will benefit the mushroom biotechnology and related research.

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