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Efficient transformation and expression of *gfp* gene in the edible mushroom *Pleurotus nebrodensis*

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Abstract

An efficient transformation method mediated by PEG-protoplasts was developed for the newly commercial edible mushroom *Pleurotus nebrodensis*. Two plasmids were used to co-transform protoplasts of *P. nebrodensis*. One plasmid is pAN7-1 containing a positive selectable marker gene *hph* conferring hygromycin B resistance. Another plasmid is pBlue-GFP containing a reporter gene *gfp* conferring green fluorescent protein. PCR and Southern blot analysis showed that *hph* gene or/and *gfp* gene were integrated into the genome of *P. nebrodensis* transformants. The transformation efficiency of the positive selectable marker gene *hph* was 3 transformants per microgram of plasmid pAN7-1 DNA, which was about 30 times higher than that previously reported in thoroughly studied *Pleurotus* species such as *Pleurotus ostreatus*. The transformation efficiency of the reporter gene *gfp* was 9 transformants per microgram of plasmid pBlu-GFP DNA. The co-transformation efficiency was 23.68%. This is the first report that a "reporter" gene, green fluorescent protein gene can be successfully stably expressed in this *Pleurotus* species.

Keywords: *Pleurotus nebrodensis*; Protoplast-mediated transformation; Green fluorescent protein; GFP expression

1. Introduction

Green fluorescent protein (GFP) expression is a very useful tool in molecular analysis of filamentous fungi [1,2]. It has been successfully expressed in several ascomycetes such as *Colletotrichum acutatum* [3], *Verticillium fungicola* [4], *Acremonium chrysosporium*, and *Sordaria macrospore* [5]. And different colored fluorescent proteins have also been expressed in ascomycetes [6]. However, GFP expression seems to be difficult in basidiomycetes. It has been successfully expressed in only three basidiomycetes, *Schizophyllum commune* [7], *Agaricus bisporus* and *Coprinus cinereus* [2] so far. Transgene expression in basidiomycetes appears to be hampered by a number of factors. In the model species *Schizophyllum commune*, transforming gene is inactivated by preferential methylation [8], AT-rich sequences inactivate gene expression [9,10], and introns are needed for mRNA accumulation to occur [7,10]. Therefore, to date, the stable expression of GFP in basidiomycete *Pleurotus* species has not been successful.

The basidiomycete, *Pleurotus nebrodensis* (Inzengae) Quél. is a commercially cultivated edible mushroom in Asian countries. It was successfully domesticated in the 1990s in northwest China. As its especial properties such as big fruiting bodies and delicious taste and distinct non-volatile components [11], its production has been steeply increasing since its successful domesticated cultivation. The annual production of *P. nebrodensis* fruiting body exceeded thousand tons in the last several years, for exam-

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ple, over 52,200 tons of its fruiting bodies were produced in 2003 in China alone [12]. However, the application of biotechnology such as protoplast regeneration, genetic transformation and gene expression to this commercially significant edible mushroom has not been reported so far. In this study, we developed a protoplast-mediated transformation method for *P. nebrodensis* for the purpose of commercial cultivation strain improvements, and GFP was stably expressed in this basidiomycete edible fungus.

2. Materials and methods

2.1. Mushroom strain and culture conditions

Pleurotus nebrodensis strain Bai-Ling number 1 was purchased from Sanming Mycological Institute (Sanming, Fujian Province, China) and it is a commercial cultivation strain in China. It was grown at 25 °C on PDSA medium (20% potato, 2% dextrose, 0.3% KH_2PO_4, 0.15% $MgSO_4$, 0.0005% vitamin B1, 2% agar) and kept at 4 °C. Vegetative cultures of mycelia were conducted in PDSB medium (the PDSA medium without agar) at 25 °C for 1 week. Transformants were screened on the PDSA medium with 80 μg/ml of hygromycin B. The fruiting bodies of transformants were produced on the cultivation medium containing 415 g of cotton seed hull, 75 g of wheat bran, 15 g of quicklime and 65% of water content in a plastic cultivation-bag with a size of 10 cm × 33 cm. The cultures for the fruiting bodies of transformants firstly were incubated for mycelium growth at 25 °C in the dark for 40–50 days and then were induced for primordium formation at 4–6 °C in light for 10–15 days, finally were incubated for fruiting body growth at 15–20 °C in weak light and over 85% of humidity with a 1 cm layer of sand covered on the cultivation medium. The fruiting bodies were harvested just before expansion (sporulation).

2.2. Plasmids

The plasmid pAN7-1 containing the selectable marker gene *hph* conferring resistance to hygromycin B derived from *Escherichia coli* and the promoter *gpd* and the terminator *trp*C derived from *Aspergillus nidulans* [13], the plasmid pBlue-GFP containing the reporter gene *gfp* conferring green fluorescent protein expressed in mushrooms and the promoter *gpd* derived from *A. bisporus* [2] were used as the expression vectors for the co-transformation of protoplasts of *P. nebrodensis*. The plasmids pAN7-1 and pBlue-GFP were kindly provided by Prof. Lorna A. Casselton at the University of Oxford and Dr. Michael P. Challen at Horticulture Research International, the University of Warwick, UK.

2.3. Protoplast preparation and co-transformation of *P. nebrodensis*

One gram of mycelium of *P. nebrodensis* grown in PDSB liquid medium for 7 days was harvested by filtration through a nylon mesh. After being washed in 0.6 M of $MgSO_4$ for two times, the mycelia were resuspended in 3 ml of lysis buffer containing 1.5% lywallzyme (Guangdong Institute of Microbiology) and 0.6 M $MgSO_4$, then incubated at 32 °C for 2.5 h with gently shaking for protoplast release. Protoplasts were purified by filtration through a glass injector with a layer of 1 mm of loose absorbent cotton and collected by centrifugation at 2000*g* for 20 min at 4 °C and washed twice with 3 ml MM buffer containing 0.5 M mannitol and 50 mM maleic acid buffer (pH 5.5). Finally protoplasts were resuspended in 2–3 ml of MMC buffer (0.5 M mannitol, 50 mM maleic acid buffer with pH 5.5, 5 mM $CaCl_2$) to a concentration of 10^8–10^9 protoplasts ml^{-1}.

For co-transformation, 3 μg of plasmid pAN7-1 and 3 μg of plasmid pBlue-GFP, 12.5 μl of PTC buffer (25% PEG4000, 10 mM Tris–HCl at pH 7.5, 25 mM $CaCl_2$) were added to 50 μl of chilled protoplast suspension and mixed well. Then the mixture was kept on ice for 20 min; then 0.5 ml of PTC buffer was added to the mixture and mixed gently, followed by incubation for 5 min at room temperature. Then the protoplast mixture was ready for plating on the regeneration and screening medium.

2.4. Screening of transformants

Screening of *P. nebrodensis* transformants was performed with a sandwich method. After co-transformation, the protoplast mixture was diluted with 1 ml STC buffer (18.2% sorbitol, 10 mM Tris–HCl at pH 7.5, 25 mM $CaCl_2$) and plated on the regeneration medium (PDSA plus 1.0 M sorbitol). After regeneration culture for 24 h at 25 °C, each plate was added with 20 ml screening medium (PDSA plus 0.8 M sorbitol, 80 μg/ml hygromycin B, 0.8% agar) and incubated at 25 °C in dark for 2 weeks. Putative transformants appeared on the screening medium were subjected to a further five-round subculture on PDSA medium containing 80 μg/ml hygromycin B for screening of stable transformants.

2.5. Characterization of transformants

Genomic DNA was isolated from mycelia of the putative stable transformants and non-transformed control of *P. nebrodensis* by the fungal DNA extraction (FDE) method. One gram of mycelium was crushed in liquid nitrogen to powder and digested in 10 ml TESN buffer (50 mM Tris–HCl at pH 7.5, 100 mM EDTA at pH 8.0, 0.5% SDS, 300 mM NaOAc at pH 5.2) at 68 °C for 1 h. After the addition of 3.5 ml 3 M NaOAc (pH 5.2) and incubation on ice for 20 min, the digestion mixture was centrifuged at 8000*g* for 20 min at 4 °C. The DNA in supernatant was extracted by phenol/chloroform extraction method.

Characterization of transformants by PCR was performed with *gfp*-specific primer GFP1 (5′-GGC CAC AAG TTC AGC GTG TC-3′) and GFP2 (5′-AGC TCG TCC ATG CCG AGA GT-3′) for detecting the integration of *gfp* gene, with *hph*-specific primer HPH1 (5′-AGC GTC TCC GAC CTG ATG-3′) and HPH2 (5′-CGA CGG ACG

CAC TGA CGG-3′) for detecting the integration of *hph* gene, respectively. Genomic DNAs from putative transformants and non-transformed recipient host (as negative control) were used as PCR amplification templates.

Positive putative transformants detected by PCR with *gfp*-specific primers were subjected to further characterization by Southern blot analysis. Of the 15 μg genomic DNA from putative transformants and non-transformed recipient host (negative control) were digested with *Hin*dIII (Bio-labs) overnight at 37 °C and electrophoresed for separation in a 0.8% agarose gel. Of the 50 pg plasmid pBlue-GFP was digested with *Nco*I (Bio-labs) and included in the electrophoresis separation as the positive control. DNA was transferred from agarose gel to Hybond N+ nylon membrane (Amersham) by the method recommended by the manufacturer. DNA probe labeling (*gfp* gene as a probe), hybridization, and signal detection were performed using the DIG system according to the manufacturer's instructions (Roche Applied Science).

2.6. Detection of GFP expression in P. nebrodensis transformants

Positive transformants characterized by PCR with *gfp*-specific primers and by Southern blot analysis with *gfp*-specific probe were detected for the GFP expression on a laser scanning confocal microscope (Leica TCS SP5, Germany). The mycelia of transformants and non-transformed recipient host (negative control) for GFP expression and detection were grown on cover glass slides in PDSA plates at 25 °C for 5 days. Fluorescence imaging of mycelium samples was obtained using 488 nm excitation line and a band-pass BP505-550 emission filter. Mycelium samples from multiple rounds of sub-culture and isolated from fruiting bodies were also used for the detection of GFP expression stability.

3. Results and analysis

3.1. Co-transformation with plasmid pAN7-1 and pBlue-GFP and transformant screening procedure

Protoplasts of *P. nebrodensis* treated with plasmid pAN7-1 and pBlue-GFP in the presence of PEG 4000/$CaCl_2$ were firstly incubated on the regeneration medium without hygromycin B for 24 h at 25 °C for their recovery growth and *hph* gene expression. If treated protoplasts were directly plated on the screening medium containing hygromycin B without this pre-screening recovery incubation, few even none transformants were obtained in the courses of subsequent screenings. After this pre-screening recovery incubation, recovering protoplasts (should be cells at this time) were then subjected to be screened for drug resistance on the sandwich regeneration plates containing hygromycin B.

After incubation at 25 °C for 11–15 days, a number of germinating protoplasts were observed on the selective plates and no such germination was observed when the protoplasts were treated with no DNA. In successive incubations, most of the germinating protoplasts stopped growing when the diameter of the colony reached 1–2 mm. The other continuously growing colonies were transferred to selective PDSA plates containing 80 μg/ml hygromycin B. After selection for five rounds, only a few tested strains grew on the selective PDSA plates (Fig. 1, part of the data shown). These strains were considered as putative transformants and taken for further analysis. All of 38 putative transformants were obtained from four times of co-transformation experiments in this study. The average transformation efficiency was about 3 transformants per microgram of plasmid pAN7-1 DNA.

3.2. PCR detection of hph gene and gfp gene in transformants

Genomic DNAs of 38 putative transformants and non-transformed host were used as templates for PCR detection with *hph*-specific primers and *gfp*-specific primers, respectively, to confirm the integration of *hph* gene and *gfp* gene. PCR products which should be 482 bp for *hph* gene appeared from all of 38 putative transformant DNA templates but not from non-transformed recipient DNA template (Fig. 2). The result showed that *hph* gene was integrated into the genomes of all 38 transformants. PCR products as expected 630 bp for *gfp* gene appeared from 9 transformant DNA templates, but not from other 29 putative transformant DNA templates and non-trans-

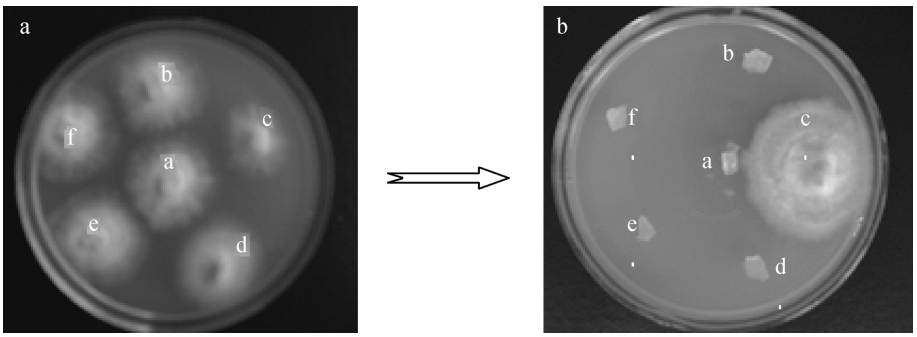

Fig. 1. Putative transformants growing on the selective PDSA medium containing 80 μg/ml hygromycin B. (a) Second round of selection; (b) fifth round of selection.

formed recipient DNA template (Fig. 3). The result indicated that *gfp* gene was integrated into the genomes of 9 transformants. Therefore, the co-transformation efficiency of plasmid pAN7-1 and pBlue-GFP was 23.68%. This value was close to 25% co-transformation efficiency of *Coprinus cinereus* that was reported by Burns et al. [2].

3.3. Southern blot analysis of transformants

Southern blot analysis was performed on nine positive transformants detected by PCR with *gfp*-specific primers with the *gfp* gene as the probe to confirm the *gfp* gene integration into the genomes of transformants. The result showed that DNA samples from eight transformants gave strong or faint specific hybridization signals (very faint hybridization signals in lane 3 and lane 9) but DNA samples from one of transformants and non-transformed host did not give any hybridization signals (Fig. 4). This indicated that *gfp* gene was incorporated into the genomes of eight transformants (transformants Tpn3, Tpn8, Tpn9, Tpn14, Tpn18, Tpn20, Tpn26, and Tpn35). No hybridization signal was found in the DNA sample from transformant Tpn25 even though PCR detection was positive for this transformant.

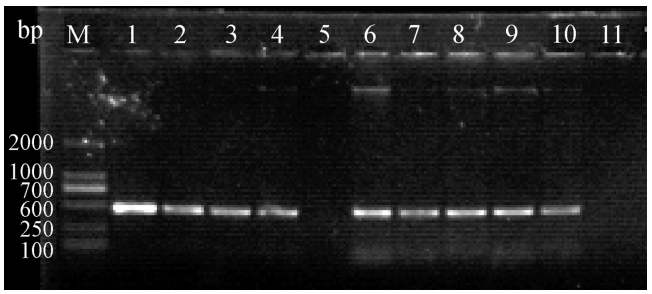

Fig. 2. Confirmation of the integration of plasmid pAN7-1 into transformant genomic DNAs by PCR analysis with *hph*-specific primers using different DNA sources. PCR products should be 482 bp for *hph* gene. M, DNA DL2000 ladder; 1, plasmid pAN7-1 as positive control; 2–4 and 6–10, transformants Tpn1, Tpn2, Tpn3, Tpn4, Tpn5, Tpn6, Tpn7, and Tpn8, respectively; 5, ddH_2O without any DNA as template as the negative control; 11, non-transformed host as the negative control.

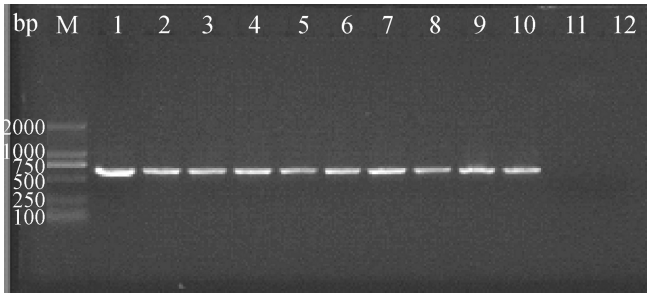

Fig. 3. Confirmation of the integration of plasmid pBlue-GFP into transformant genomic DNAs by PCR analysis with *gfp*-specific primers using different DNA sources. PCR products should be 630 bp for *gfp* gene. M, DNA DL2000 ladder; 1, plasmid pBlue-GFP as positive control; 2–10, transformants Tpn3, Tpn8, Tpn9, Tpn14, Tpn18, Tpn20, Tpn25, Tpn26, and Tpn35, respectively; 11, non-transformed host as negative control; 12, ddH_2O without any DNA as template as one of negative control.

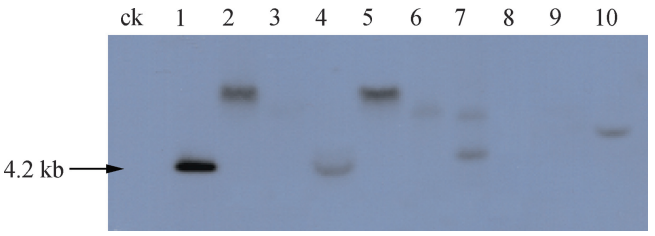

Fig. 4. Southern blot analysis of *P. nebrodensis* transformants. *Hin*dIII-digested genomic DNA (15 μg) samples isolated from transformants and non-transformed host were probed with *gfp* gene fragment. ck, DNA isolated from the non-transformed host strain as the negative control; lane 1, plasmid pBlue-GFP (50 pg) as the positive control; lanes 2–10, DNA isolated from transformants Tpn3, Tpn8, Tpn9, Tpn14, Tpn18, Tpn20, Tpn25, Tpn26, and Tpn35, respectively.

Southern hybridization patterns with different numbers of bands and strong or faint signals demonstrated that single copy or multiple copies of *gfp* gene were integrated into the genomes of *P. nebrodensis* transformants at different chromosomal sites (Fig. 4).

3.4. GFP expression in P. nebrodensis transformants

All of 38 transformants of *P. nebrodensis* were microscopically detected for GFP expression in their mycelia. The result showed that five transformants (Tpn3, Tpn9, Tpn14, Tpn20, and Tpn35) strongly expressed GFP in their mycelia (Fig. 5). But GFP expression was not detectable in any other 33 transformants even though the GFP DNA presence in the genome of these transformants was confirmed by PCR and/or Southern blot hybridization (not all results presented).

Five transformants (Tpn3, Tpn9, Tpn14, Tpn20, and Tpn35) that strongly expressed GFP in their mycelia were cultivated on cotton seed hull medium using plastic culture bags in order to obtain their fruiting bodies. The fruiting body morphology of five transformants was similar to that of the non-transformed host strain (results not presented). Mycelia from multiple rounds of subculture and isolated from fruiting bodies of five transformants were detected for GFP stable expression. The result showed that GFP expression was stable in mycelia during the different rounds of subculture and isolated from fruiting bodies of five transformants (results not presented). This result is similar to the observed results for *A. bisporus* and *Coprinus cinereus* [2], but different from the result for *A. bisporus* [14].

4. Discussion

This is the first report of the development of a protoplast-mediated transformation method for *P. nebrodensis*. Although genetic transformation procedures have been developed for *Pleurotus ostreatus*, their transformation efficiency was very low [15–21]. In this study, we developed a

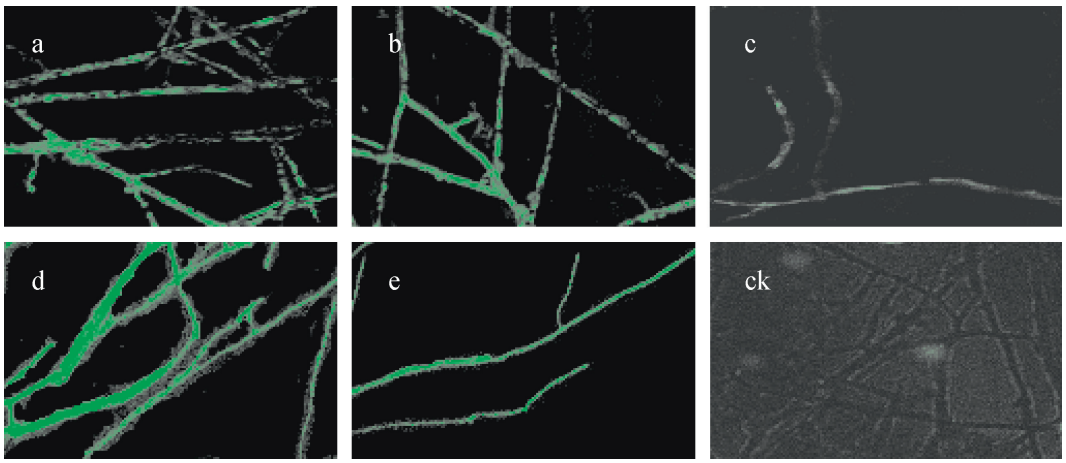

Fig. 5. Expression of GFP in *P. nebrodensis* co-transformed with plasmid pAN7-1 and pBlue-GFP. a–e, Mycelia of transformants Tpn3, Tpn9, Tpn14, Tpn20, and Tpn35 expressing green fluorescence viewed using a confocal microscope illuminated with a UV light. ck, Mycelia of non-transformed host strain without the expression of green fluorescence.

reliable transformation method for a novel commercial *Pleurotus* species, *P. nebrodensis* with relatively high transformation efficiency of 3 transformants per microgram of plasmid pAN7-1 DNA. This value was low when compared with the *Trametes versicolor* that 25–50 transformants per microgram of plasmid DNA was obtained [22], which was transformed with the hygromycin B resistance gene using the REMI transformation. But this value was high when compared with *A. bisporus* and *P. ostreatus* that their transformation efficiencies were 0.1–0.5 transformants and 0.1 transformants per microgram of plasmid DNA [21,23].

No hybridization signal was found in the DNA sample from transformant Tpn25 even though PCR detection was positive for this transformant. This might be due to the *gfp* gene loss from the genome of transformant Tpn25 after multiple rounds of subculture. This might also be due to incomplete DNA sample digestion by restriction enzyme or incomplete DNA sample transfer to nylon membrane during Southern blotting process. In our experiments, genomic DNA isolated from *P. nebrodensis* transformant mycelia cultivated in PDSB liquid medium contained a plentiful of polysaccharides which were hardly removed from DNA samples. This might interfere with DNA sample digestion, migration and transfer during Southern blotting.

This is also the first report of GFP stable expression in a *Pleurotus* species, *P. nebrodensis*. Previous attempts to express GFP in basidiomycetes were successful in only three cases without a *Pleurotus* species even thoroughly studied *P. ostreatus* [2]. In this study, GFP stable expression was detectable in 5 transformants, but not in other 3 transformants even though the *gfp* gene presence in the genome of these three transformants was confirmed by Southern blotting. This result may suggest that transgene expression of GFP in some *P. nebrodensis* transformants was inactivated by some factors, similar to those results in basidiomycete *Schizophyllum commune* [8–10], *Coprinus cinereus* and *A. bisporus* [2].

The work reported in this paper will be useful for further transgenic manipulation of *P. nebrodensis*, facilitating efforts to improve the value of this commercially significant and promising *Pleurotus* species and perhaps other commercially important cultivated *Pleurotus* species such as *P. ostreatus*, *P. ferulae*, *P. eryngi*, *P. pulmonarius*, and *P. abalones*.

Acknowledgements

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References

[1] Lorang JM, Tuori RP, Martinez JP, et al. Green fluorescent protein is lighting up fungal biology. Appl Environ Microbiol 2001;67:1987–94.
[2] Burns C, Gregory KE, Kirby M, et al. Efficient GFP expression in the mushroom *Agaricus bisporus* and *Coprinus cinereus* requires introns. Fungal Genet Biol 2005;42:191–9.
[3] Horowitz S, Freeman S, Sharon A. Use of green fluorescent protein-transgenic strains to study pathogenic and nonpathogenic lifestyles in *Colletotrichum acutatum*. Phytopathology 2002;92:743–9.
[4] Amey RC, Athey-Pollard A, Burns C, et al. PEG-mediated and *Agrobacterium*-mediated transformation in the mycopathogen *Verticillium fungicola*. Mycol Res 2002;106:4–11.
[5] Pöggeler S, Masloff S, Hoff B, et al. Versatile eGFP reporter plasmids for cellular localization of recombinant gene products in filamentous fungi. Curr Genet 2003;43:54–61.
[6] Bourett TM, Sweigard JA, Czymmek KJ, et al. Reef coral fluorescent proteins for visualizing fungal pathogens. Fungal Genet Biol 2002;37:211–20.
[7] Lugones LG, Scholtmeijer K, Klootwijk R, et al. Introns are necessary for mRNA accumulation in *Schizophyllum commune*. Mol Microbiol 1999;32:681–9.
[8] Mooibroek H, Kuipers AGJ, Sietsma JH, et al. Introduction of hygromycin B resistance into *Schizophyllum commune*-preferential methylation of donor DNA. Mol Gen Genet 1990;222:41–8.

[9] Schuren FHJ, Wessels JGH. Expression of heterologous genes in *Schizophyllum commune* is often hampered by the formation of truncated transcripts. Curr Genet 1998;33:151–6.
[10] Scholtmeijer K, Wösten HAB, Springer J, et al. Effect of introns and AT-rich sequences on expression of the bacterial hygromycin B resistance gene in the basidiomycete *Schizophyllum commune*. Appl Environ Microbiol 2001;67:481–3.
[11] Guo LQ, Lin JY, Lin JF. Non-volatile components of several novel species of edible fungi in China. Food Chem 2007;100(2):643–9.
[12] Chang ST. Witnessing the development of the mushroom industry in China. In: Proceedings of the fifth international conference on mushroom biology and mushroom products, Shanghai, China, April 8–12; 2005. p. 3–19.
[13] Punt PJ, Oliver RP, Dingemanse MA, et al. Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. Gene 1987;56(1):117–24.
[14] Chen X, Stone M, Schlagnhaufer C, et al. A fruiting body tissue method for efficient *Agrobacterium*-mediated transformation of *Agaricus bisporus*. Appl Environ Microbiol 2000;66(10):4510–3.
[15] Peng M, Singh NK, Lemke PA. Recovery of recombinant plasmids from *Pleurotus ostreatus* transformants. Curr Genet 1992;22:53–9.
[16] Peng M, Lemke PA, Shaw JJ. Improved conditions for protoplast formation and transformation of *Pleurotus ostreatus*. Appl Microbiol Biotechnol 1993;40(1):101–6.
[17] Yanai K, Yonekura K, Usami H, et al. The integrative transformation of *Pleurotus ostreatus* using bialaphos resistance as a dominant selectable marker. Biosci Biotechnol Biochem 1996;60(3):472–5.
[18] Jia JH, Buaswell JA, Peberdy JF. Transformation of the edible fungi, *Pleurotus ostreatus* and *Volvariella volvacea*. Mycol Res 1998;102(7):876–80.
[19] Kim BG, Magae Y, Yoo YB, et al. Isolation and transformation of uracil auxotrophs of the edible basidiomycete *Pleurotus ostreatus*. FEMS Microbiol Lett 1999;181:225–8.
[20] Honda Y, Matsuyama T, Irie T, et al. Carboxin resistance transformation of the homobasidiomycete fungus *Pleurotus ostreatus*. Curr Genet 2000;37(3):209–12.
[21] Irie T, Honda Y, Hirano T, et al. Stable transformation of *Plearotus ostreatus* to hygromycin B resistance using *Lentinus edodes* GPD expression signals. Appl Microbiol Biotechnol 2001;56:707–9.
[22] Kim K, Leem Y, Kim K, et al. Transformation of the medicinal basidiomycete *Trametes versicolor* to hygromycin B resistance by restriction enzyme mediated integration. FEMS Microbiol Lett 2002;209:273–6.
[23] van de Rhee MD, Graca PMA, Huizing HJ, et al. Transformation of the cultivated mushroom, *Agaricus bisporus*, to hygromycin B resistance. Mol Gen Genet 1996;250:252–8.