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Heterologous expression of EGFP in enoki mushroom *Flammulina velutipes*

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ABSTRACT. Heterologous expression in *Flammulina velutipes* using mycelia-based electroporation was reported. The enhanced green fluorescent protein (EGFP) was expressed and remained stable after multiple rounds of subculture without selection pressure. Using the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter containing its first intron, *egfp* could be successfully expressed during the life cycle of *F. velutipes*, including primordia, mature fruiting bodies, basidiospores, and monokaryons produced from meiosis. This is the first report of *egfp* gene expression in the complete life cycle of an edible mushroom. The presence of extracellular EGFP was shown by fluorescent microscopy and western hybridization. Southern analysis of dikaryon and its green progeny showed the *egfp* integrated into genome, and all signals of progeny coincided with those of parental dikaryon. These results revealed that the integrated DNA was stable during meiosis. The promoter deletion analysis showed that the region of 336 bp upstream from the transcription start point was sufficient for the function of the *F. velutipes gpd* promoter. This mycelia-based electroporation procedure provides a useful tool for the molecular biology of mushrooms.

Keywords: EGFP; Electroporation; *Flammulina velutipes*; Glyceraldehyde-3-phosphate dehydrogenase; Heterologous expression; Transformation.

INTRODUCTION

Modern biotechnology relies on genetic engineering techniques to enable the expression of heterologous genes in various organisms. The application of biotechnology to cultivated mushrooms was hampered until genetic transformation systems had been developed. With respect to molecular breeding and the potential of using mushrooms as expression hosts, researchers have put much effort into the development of genetic transformation systems for edible mushrooms. Most protocols involved protoplast preparation and electroporation (Noel and Labarere, 1994; Van de Rhee et al., 1996) or the treatment of $CaCl_2$ or polyethylene glycol (Ogawa et al., 1998; Sato et al., 1998; Hirano et al., 2000). However, the difficulty of regenerating sufficient protoplasts limited the application of these techniques to other edible mushrooms. Other research groups have demonstrated the potential of using *Agrobacterium tumefaciens*-mediated transformation in the genetic modification of mushrooms (De Groot et al., 1998; Chen et al., 2000; Mikosch et al., 2001; Hanif et al., 2002; Combier et al., 2003; Godio et al., 2004; Burns et al., 2005; Kemppainen et al., 2005). Despite a recent review discussing the "promiscuity" of the *Agrobacterium* system (Lacroix et al., 2006), further development was required before it could be applied to other mushrooms.

Although several cases have been reported regarding the expression of heterologous genes (Ma et al., 2001; Ogawa et al., 1998) or increased homologous gene expression (Alves et al., 2004; Lugones et al., 1999) in basidiomycetes, in the scope of mushrooms, only homologous manganese peroxidase (Irie et al., 2001; Tsukihara et al., 2006) and two reporter genes β-glucuronidase (Sun et al., 2002) and green fluorescent protein (Burns et al., 2005) were expressed at the stage of mycelium. To date, reporter gene expression throughout the life cycle of edible mushroom—including primordia, mature fruiting bodies, basidiospores, and monokaryons produced from meiosis—has not been reported.

In our previous study, a transformation procedure based on basidiospore electroporation for *Flammulina velutipes* and *Lentinula edodes* was developed (Kuo et al., 2004; Kuo and Huang, 2008). This method eliminated the problem of protoplast regeneration and the limitation of host specificity. In this study, a much simpler and reliable

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electroporation procedure for mycelial cell transformation was developed. The heterologous expression of the enhanced green fluorescent protein (*egfp*) in *F. velutipes* is reported. We also demonstrate that *egfp* was expressed in mature fruiting bodies and remained stable in monokaryons produced from meiosis.

MATERIALS AND METHODS

Strains and media

Flammulina velutipes BCRC 37086 was purchased from the Bioresources Collection and Research Center (Hsinchu, Taiwan) and grown in either PDA (Potato dextrose agar, Difco, Detroit, MI, USA) or PDB (Potato dextrose broth, Difco) at 25°C. The *Escherichia coli* DH5α (GIBCOBRL, Life Technologies, Grand Island, NY, USA) was used for DNA manipulation and grown in LB medium (Sigma Chem. Co., St. Louis, MO, USA) at 37°C.

Fruiting body development of *F. velutipes*

A medium composed of 65% sawdust and 35% rice bran was placed into a 500-ml flask and autoclaved for 1 h. Capped flasks inoculated with mycelial plugs were incubated at 25°C in the dark for 3 weeks. After vegetative mycelia were growing all through the medium, fruiting was induced by water addition, a temperature shift from 25°C to 10°C, and light exposure. To achieve the appearance of primordia and the maturation of fruiting bodies, the flasks were incubated under the induction conditions until mature fruiting bodies appeared.

Plasmid construction

The plasmid pFGH was used as a backbone (Kuo et al., 2004). Primers used to amplify reporter genes and *gpd* promoter regions (pGPD) for deletion analysis are shown in Table 1. Promoters were amplified from *F. velutipes* genomic DNA; *egfp* gene were amplified from pHygEGFP (BD Bioscience, Palo Alto, CA). The resulting plasmids (Figure 1) were used for the transformation experiments.

Transformation procedure

Transformation was modified as in a previous paper (Kuo et al., 2004). A four-day-old liquid culture of mycelia was blended with a Waring blender and then incubated overnight with gentle shaking at 25°C. Mycelial fragments were collected by centrifugation at 3,000 *g*, followed by washing with P buffer (0.02 M phosphate buffer, pH 5.8, 0.6 M mannitol) and treatment with 2 mg/ml Lysing enzymes (Sigma) for 3 h. After washing free of enzyme, 0.5 g (wet weight) mycelial fragments were mixed with plasmid DNA and subjected to electroporation. Electroporation was performed by BTX ECM 630 and 0.2-cm cuvettes (BTX, San Diego, CA, USA) with the electric pulse delivery setting of 25 μF in capacitor, 100 Ω in resistor, and 12.5 kV/cm in field strength. Transformants were selected on PDA plates containing 30 μg/ml hygromycin (Sigma).

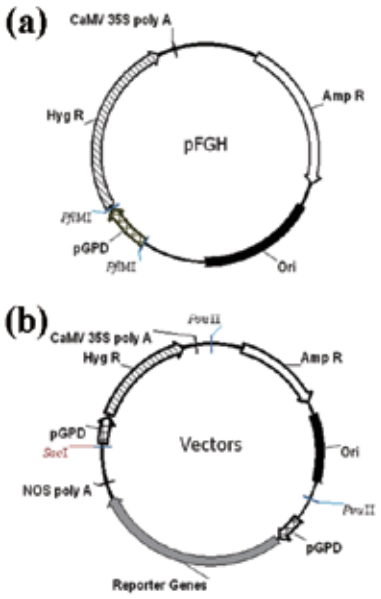

Figure 1. Organization of plasmids transformed. (a) Plasmids used in deletion analysis consisted of a pFGH vector backbone (Kuo et al., 2004). Different lengths of *pGPD* fragments were introduced by restriction enzyme *Pfl* MI; (b) Plasmid for the expression of *egfp* as reporter gene. The hygromycin resistance gene (Hyg R) and reporter gene were joined to the *gpd* promoter (*pGPD*). NOS poly A: nopaline synthase poly A signal. CaMV35S poly A: CaMV 35S poly A signal. Amp R: the ampicillin resistance gene.

Detection of EGFP in transformants

EGFP transformants were screened by a fluorescent microscope (E600, Nikon, Tokyo, Japan) using a Nikon B-2A filter (excitation filter, 450-490 nm; dichroic filter, 505 nm; barrier filter, 520 nm). Fruiting bodies of transformants and wild-type were observed using a stereo fluorescence microscope (SV11 APO/AxioCam MRc5, Carl Zeiss, Inc., Thornwood, NJ), and the fluorescent microphotographs were captured under the same exposure time.

Southern hybridization

Genomic DNA isolation and the southern hybridization procedure were described previously (Kuo et al., 2004). Labeling of the DNA probe, hybridization, and signal detection were conducted by means of a Roche DIG-probe synthesis and detection kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

Western hybridization

For western analysis of EGFP, *F. velutipes* transformants and the wild-type strain were grown for 7 days in PDB. Mycelia were collected and subsequently ground in liquid nitrogen with a mortar and pestle. A total of 50 mg mycelial powder was mixed with 1 ml protein extraction buffer (50 mM sodium phosphate, pH 7.4, 1 mM PMSF, 0.1% Triton X-100, 0.5 M NaCl) on

Table 1. Primers used in plasmids construction.

Target genes	Primers used	Usage
F. velutipes pGPD	pFG1.0-f (5' TTCGACGGGCAAGGAGGGTCAAG 3')	Forward primer for *pGPD* cloning in deletion analysis as pFGH and driving reporter genes.
	pFG0.8-f (5' CACTGTTGCGAGAGTAAG 3')	Forward primer for *pGPD* cloning in pFG0.8-hpt
	pFG0.6-f (5' TCCAGCATCTTGTTCGAG 3')	Forward primer for *pGPD* cloning in pFG0.6-hpt
	pFG0.5-f (5' TGTGCTCCAGCCAATTAG 3')	Forward primer for *pGPD* cloning in pFG0.5-hpt
	pFG0.2-f (5' GACATGATCTAGGTCGTC 3')	Forward primer for *pGPD* cloning in pFG0.2-hpt
	pFG-r (5' TTGTAGATGAGGAGATGGTAAAG 3')	Reverse primer for *pGPD* cloning in deletion analysis and driving reporter genes without the 1st intron.
	pFG-ir (5' AACTTTGACCTGTAAAATG 3')	Reverse primer for *pGPD* cloning with the 1st intron to drive *egfp*.
egfp	Egfp-f (5' GGTTAAAGTGAGCAAGGGCGAGG 3')	Forward primer for *egfp* cloning
	Egfp-r (5' ACTAGTTACTTGTACAGCTCGTCC 3')	Reverse primer for *egfp* cloning.

ice for a period of 5 min. After centrifuging at 13,000 *g* for 20 min, supernatant was collected as total cellular protein. To verify the secretion of EGFP, the extracellular culture supernatant from liquid culture and water droplets on hyphae tip from the agar plate were applied to immunoblotting. Total cellular protein and extracellular samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a PVDF sequencing membrane (Millipore, Bedford, MA, USA), using a semi-dry blotting system (Genmedika, Taipei, Taiwan). The detection was carried out with a (1:6,000) monoclonal anti-GFP living-colors peptide antibody (BD Bioscience) and with the BCIP/NBT Western detection kit (PerkinElmer, Boston, MA) as described by the manufacturers.

Deletion analysis

To determine the essential part of the 5'-flanking region of *F. velutipes gpd* gene for its promoter function; several plasmids were constructed in which the 5'-flanking region was deleted at the 5' end. The hygromycin resistance gene (*hpt*) was joined to the 80, 60, 50, or 20% length of the 5'-flanking region of *F. velutipes gpd* gene to construct pFG0.8-hpt, pFG0.6-hpt, pFG0.5-hpt, and pFG0.2-hpt, respectively. The plasmid pFGH contained the full length of the 5'flanking region of *F. velutipes gpd* gene, and pFG0-hpt contained no promoter. These resulting plasmids were used for the transformation efficiency test.

RESULTS

Transformation procedure

An electroporation procedure for *F. velutipes* transformation was reported previously (Kuo et al., 2004). Although the transformation procedure using basidiospores electroporation was more applicable than other published methods, it had some limitations, such as easy contamination during basidiospore isolation. It was also inapplicable to some other species that had difficulty in fruiting. Using the competent cell preparation described in this study, small mycelial fragments, both dikaryons and monokaryons, could be transformed as easily as germinated basidiospores. The transformation efficiency was about 5 to 20 transformants per µg DNA while no hygromycin resistant colonies were observed in the control experiment without adding plasmid DNA. There was no significant difference in growth rate or morphology between transformants and the wild type strain.

Detection of EGFP in transformants

The requirement of having intron for *egfp* expression in some basidiomycetes has been reported (Lugones et al., 1999; Ma et al., 2001; Burns et al., 2005); therefore the promoter region with or without the first intron of the *gpd* gene was cloned to test its feasibility to drive *egfp*. About 30% of *F. velutipes* transformants with the first intron showed green fluorescence (Figure 2). The expression of

egfp remained stable after multiple rounds of subculture without selection pressure. In contrast, no expression was observed for the construct without the first intron region. This result indicated the presence of 5' intron is required for the *egfp* expression in *F. velutipes*.

The expression of *egfp* was also confirmed by western hybridization as shown in Figure 3. Immunoblotting with the monoclonal anti-GFP antibody detected a 27-kDa polypeptide in positive control (Lane 1) and in total cellular protein from transformant (Lane 3) while no signal was found in the untransformed strain (Lane 2). By observing colonies on the selection agar plate using epifluorescent microscopy, it was found that water droplets on the hyphae tip emitted green fluorescence (Figure 2e, f) while water droplets of the wild type controls did not show florescent. To investigate the possibility of EGFP secretion, the water droplets on the hyphae tip and samples of liquid culture medium taken at day 3, 7, and 10 during growth were examined by immunoblotting. The presence of extracellular EGFP was shown in the Lane 4-7 of Figure 3, and the signals were increased with time (Lane 5-7, Figure 3). These results in conjugation with the epifluorescent microscopic observation showed that EGFP could secret extracellularly without secretion signal peptide.

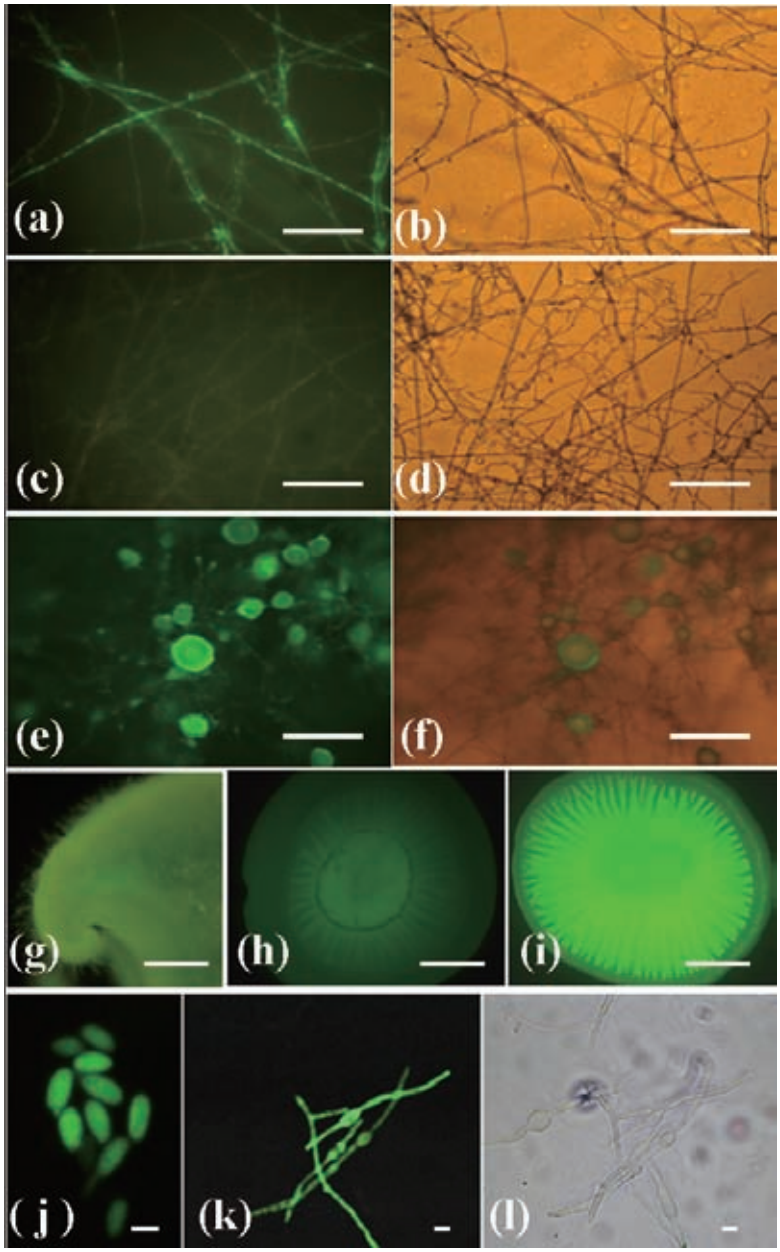

Figure 2. Expression of *egfp* in *F. velutipes* transformant. Transformant (a, b) and wild type (c, d) mycelia collected from liquid culture medium; Transformant on selection agar plate (e, f); Transformant primordia (g) and mature fruiting bodies (h, i); Isolated basidiospores from transformant (j) and mycelia after germination (k, l). Bar = 10 μm in (a-d); 40 μm in (e, f); 0.5 mm in (g); 5 mm in (h and i); 5 μm in (j, k, l).

Using dikaryotic mycelial fragments as the recipient, ten randomly selected transformants that expressed *egfp* were inoculated into the sawdust medium for fruiting body isolation. Fruiting body development was induced after vegetative mycelia grew all over the sawdust medium. Primordia appeared 3 weeks after induction. For the maturation of fruiting bodies, the bottles were incubated at 10°C and exposed to light for another 20 days. All of these transformants fructified successfully. Primordia and fruiting bodies were collected for EGFP observation by fluorescent microscopy. Primordia (Figure 2g) produced less EGFP than mature fruiting body and intense green fluorescence was found on gills (Figure 2i). Although some autofluorescence was seen in wild type fruiting body illuminated by UV light, transformed (Figure 2i) and non-transformed strains exhibited obvious differences (Figure 2h). No green fluorescence was detected during the life cycle of wild type *F. velutipes*, including the primordia, basidiospores, and monokaryons produced from meiosis (data not shown). The presence of EGFP in fruiting bodies was also confirmed by immunoblotting (Figure 3, Lane 8, 9). Basidiospores isolated from these mushrooms radiated green fluorescence before (Figure 2j) and after germination (Figure 2k). Southern analysis of one dikaryon and its nine green progeny, as illustrated in Figure 4, showed the *egfp* integrated into the genome, and all signals of progeny coincided with those of the parental dikaryon. These results revealed that the integrated DNA was stable during meiosis.

Deletion analysis of FpGPD

To determine which part of the 5'-flanking region of *F. velutipes gpd* gene was essential for its promoter function, several plasmids were constructed in which the 5'-flanking region was partially deleted at the 5' end (Figure 5). No hygromycin resistant transformants were obtained using pFG0-hph, which lacks the *gpd* promoter, while pFG0.2-hph, pFG0.4-hph, pFG0.5-hph, pFG0.8-hph, and pFGH

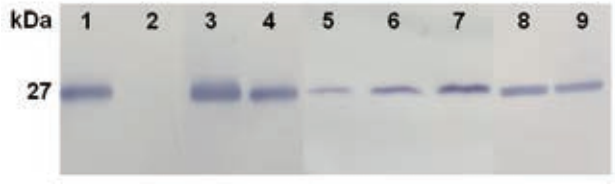

Figure 3. Western hybridization of EGFP of transformant. Lane 1: positive control of EGFP expressed in *E. coli.* Lane 2: negative control of total cellular protein from untransformed wild type *F. velutipes*. Lane 3: total cellular protein from mycelia of transformant. Lane 4: water droplets from hyphae tip of transformant on selective agar. Lane 5, 6, and 7: extracellular liquid culture supernatant of transformant sampling on day 3, 7, and 10, respectively. Lane 8, 9: protein extracted from cap and stem of mature fruiting body of transformant, respectively. Samples were fractionated on a SDS-PAGE and blotted on a PVDF sequencing membrane. Detection was carried out with a (1:6000) monoclonal anti-GFP living-colors peptide antibody.

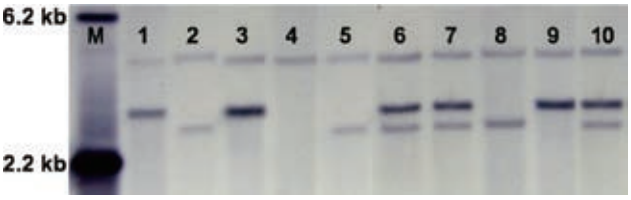

Figure 4. Southern-blot analysis of one dikaryon transformant and its nine green progeny. Lane 10: genomic DNA of dikaryon transformant; Lane 1-9: 9 progeny from dikaryon transformant in Lane 10. *Sac*I-digested genomic DNA was probed with the DIG-labeled *egfp* sequence. M, DNA molecular size markers (kilobases).

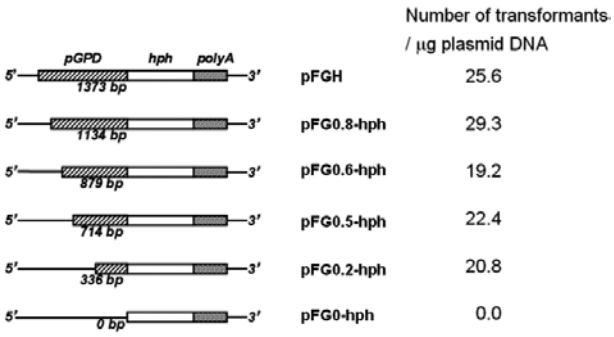

Figure 5. Deletion analysis of *F. velutipes pGPD*. The hygromycin resistance gene (*hpt*) was joined to the 80%, 60%, 50%, or 20% length of the putative *gpd* promoter (*pGPD*) to construct pFG0.8-hph, pFG0.6-hph, pFG0.5-hph, and pFG0.2-hph, respectively. pFGH contains the full length of the 5' flanking region of the *F. velutipes gpd* gene, and pFG0-hph contains no promoter. Transformation efficiency of each construct represents the averages of three replicates. *poly A*: nopaline synthase poly A signal.

were able to yield transformants. There was no significant difference in the total number of transformants for plasmids containing different lengths of the 5'-flanking region. These results suggested that the region of 336 bp upstream from the transcription start point was sufficient for the function of the *F. velutipes gpd* promoter.

DISCUSSION

In comparison with other transformation protocols requiring protoplast regeneration, transformation using mycelial fragments retained the advantages of the basidiospores-based procedure but eliminated the problem of easy contamination during basidiospore isolation. Applying mycelia-based protocol to dikaryons, one can even produce transformants for fruiting without mating of compatible monokaryons. This protocol could be employed not only in *F. velutipes*, but also in other edible mushrooms such as *Lentinula edodes, Hypsizygus marmoreus*, and *Pleurotus ostreatus* (data not shown).

The intron was required for *egfp* expression in *F. velutipes*, and this was consistent with other homobasidiomycetes. For *Schizophyllum commune*, the intron immediately

downstream of the 3' end of the *gfp* coding region was required (Lugones et al., 1999) while another intron-*gfp* coding region was used in *Phanerochaete chrysosporium* (Ma et al., 2001), *A. bisporus*, and *Coprinus cinereus* (Burns et al., 2005). However, no visualization of GFP-associated fluorescence in *F. velutipes* was observed when other introns were used except the first intron of *gpd* gene.

According to observation of epifluorescent microscopy, dikaryotic transformants expressing stronger green fluorescence had a better chance to produce green-fluorescent monokaryons. These might be attributed to the difference of gene dosage in dikaryons and the distribution of *egfp* in monokaryons, but no clear relationship between the diversity of integrated DNA and heterologous gene expression was found. The green fluorescence of the water droplets on the transformant hyphae tip might be attributable to the non-classical secretion pathway of correctly folded EGFP in *F. velutipes*. The non-classical secretion of GFP without signal peptide was also detected in Chinese hamster ovary cells. However, the secreted GFP was not fluorescent due to incorrect folding (Tanudji et al., 2002).

One typical TATA box (TATAAAA) and two CAAT boxes (CCAAT) were located in the 5'-flanking region of the *gpd* promoter of *F. velutipes*, but the CAAT boxes were located farther upstream from the initiating ATG codon than other published basidiomycetes *gpd* genes (Kuo et al., 2004). According to the results of deletion analysis, the region of 336 bp upstream from the transcription start point containing no CAAT box was sufficient for the function of the *F. velutipes gpd* promoter, indicating that the CAAT boxes were not involved in regulation of the *F. velutipes gpd* gene.

In this work, we reported that the *egfp* was faithfully expressed in *F. velutipes*. Furthermore, *egfp* could be successfully expressed throughout the life cycle of *F. velutipes*, including in primordia, mature fruiting bodies, basidiospores, and monokaryons produced from meiosis. This is the first report of *egfp* expression in the complete life cycle of an edible mushroom. Beyond the present work, *hpt* and *egfp* have been expressed successfully in other edible mushrooms such as *L. edodes, H. marmoreus*, and *P. ostreatus*. We believe that the procedure developed in this study could be applied to other basidiomycetes and provide a useful tool for the molecular biology of mushrooms.

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本研究以小片段的營養菌絲爲材料，利用電穿孔完成金針菇異源表達。綠色螢光蛋白質可在金針菇表達，在無選殖壓力的環境下繼代培養數次仍維持穩定。利用甘油醛 -3- 磷酸脫氫酶基因啓動子及其第一個內含子，綠色螢光蛋白質可在金針菇生活史中的每個階段表現，包括基原體、成熟之子實體、擔孢子及經減數分裂所生成之單核體。此爲首次綠色螢光蛋白質可以在食用菇完整生活史表達之報告。雙核菌株及其減數分裂所生成單核體之南方氏雜合分析訊號相符，顯示外來基因在減數分裂後仍能穩定存在。金針菇啓動子刪除分析顯示僅須轉錄啓始點上游 336 鹽基對區域即足夠具有啓動子功能。

關鍵詞：金針菇；甘油醛 -3- 磷酸脫氫酶；異源表達；轉形；電穿孔；綠色螢光蛋白質。