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Note

Transformation of the mushroom species *Hypsizigus marmoreus*, *Flammulina velutipes*, and *Grifola frondosa* by an *Agrobacterium*-mediated method using a universal transformation plasmid

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ABSTRACT

Agrobacterium tumefaciens-mediated transformation (AMT) was successfully applied to mycelia of the 3 economically important mushrooms *Hypsizigus marmoreus*, *Flammulina velutipes*, and *Grifola frondosa*. We used the hygromycin B resistance gene (*hph*) under the control of the *Cryptococcus neoformans* actin promoter. Eighty-six resistant strains of *H. marmoreus*, 4 of *F. velutipes*, and 2 of *G. frondosa* were obtained. All transformants were highly resistant to hygromycin B, suggesting that the *C. neoformans* actin promoter has a potential universal promoter activity in basidiomycetes. Southern analysis revealed random but single integration of the *hph* gene.

Agrobacterium tumefaciens-mediated transformation (AMT) has been used for genetic engineering of variety of plants. Since Bundock et al. (1995) succeeded in transformation of *Saccharomyces cerevisiae* by using AMT, this method has come to be used for fungi (De Groot et al. 1998). Transformation by AMT has been reported in various important fungi, including the mushroom species *Agaricus bisporus* (Chen et al. 2000), *Hypholoma sublateritium* (Godio et al. 2004), *Suillus grevillei* (Murata et al. 2006a,b), and *Flammulina velutipes* (Cho et al. 2006; Okamoto et al. 2010).

To develop a transformation system for basidiomycetous fungi—especially for mushroom species—several promoters for driving the expression of the marker gene must generally be evaluated (Chen et al. 2000; Murata et al. 2006a,b). The

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Aspergillus nidulans trpC promoter is well known to be effective in driving the expression of selectable markers in a wide range of ascomycetous fungi, but it does not work well in some basidiomycetous fungi (Godio et al. 2004; Okamoto et al. 2010).

Here, we developed a transformation method for 3 economically important mushroom species, *Hypsizigus marmoreus*, *F. velutipes*, and *Grifola frondosa*, by using an AMT method. In these experiments, we commonly used the transforming plasmid pPZP-HYG2 (Walton et al. 2005), which contains a hygromycin resistance gene (*hph*) under the regulation of the actin promoter of *Cryptococcus neoformans*. Our results indicate that the actin promoter of *C. neoformans* has potential as a universal promoter for driving the expression of selectable markers in a wide range of mushroom species.

A. tumefaciens EHA105 (Hood et al. 1993) harboring pPZP-HYG2 (gift from Dr. Alexander Idnurm; Walton et al. 2005) was used for the mushroom transformation. *Agrobacterium* cells carrying pPZP-HYG2 were inoculated into LB liquid medium containing 200 μg/ml kanamycin and grown at room temperature using a rotary shaker to the mid-exponential growth phase. The culture was centrifuged, and the supernatant was removed. The bacterial cells were diluted to an optical density (wavelength 660 nm) of 0.2 in induction medium (IM) (Bundock et al. 1995).

H. marmoreus was grown in complete medium (CM; Tanaka et al. 1991) at room temperature using a rotary shaker for about 2 wk, *F. velutipes* was grown under the same conditions for about a week and *G. frondosa* was grown for about 20 d. Then the medium was removed by centrifugation. The mycelia were resuspended in 1 ml IM medium and homogenized with an Ultra-Turrax T25 homogenizer (IKA Werke, Satufen, FRG).

Agrobacterium cells and fungal suspensions were mixed in a 1:1 (v/v) ratio. An aliquot of 200 μl of the mixture was spread on each autoclaved cellulose acetate filter (35 mm diameter; Y008A035A; Toyo Roshi Kaisha, Tokyo, Japan) on the IM plate and incubated at 28 °C for 2–4 d. After the co-cultivation, the filters were transferred to selection medium (glucose 10 g/l, yeast extract 1 g/l, trypton 1 g/l, agar 12 g/l) supplemented with 50 μg/ml cefotaxime and 50 μg/ml tetracycline hydrochloride to counter-select *Agrobacterium* cells and 50 or 100 μg/ml hygromycin B to select for fungal transformants.

To test the resistance of strains to hygromycin B, we grew the resistant strains or wild types of *F. velutipes*, *H. marmoreus*, and *G. frondosa* on modified Hamada's medium (glucose 20 g/l, yeast extract 2 g/l, KH_2PO_4 1 g/l, agar 12 g/l) containing 0, 10, 25, 50, 100, or 200 μg/ml hygromycin B. After 11 days, the radii of the colonies of *H. marmoreus* were measured. The size of the colonies of *Flammulina vertipes* and *G. frondosa* were measured after 6 d and 22 d respectively. The measured colony size means the length from inoculum disc edge to colony edge.

For molecular analysis of transformants, genomic DNA was extracted from mycelia of transformants and wild-type strains, according to our previously published methods (Nakada et al. 1994; Izumitsu et al. 2009, 2012). DNA handling and digestion and gel electrophoresis were conducted according to standard methods (Sambrook et al. 1989). The existence of *hph* was assessed by PCR analysis using the primers HPH f1 (5′-TTCGACAGCGTCTCCGACCTGATGC-3′) and HPH r1 (5′-TGCTGCTCCATACAAGCCAACCACG-3′). The existence of actin promoter of *C. neoformans* in upstream region of the *hph* gene was also assessed by PCR analysis using the different primer set [ACTPRO f1 (5′-CCATTGGAT CATGGGTGCTAGG-3′) and HPH r2 (5′-ATGCAATAGGTCAG GCTCTCGC-3′)]. The PCR products were analyzed by TAE-1% agarose gel electrophoresis.

For Southern blot analysis, genomic DNA was digested with *Hin*dIII. DNA fragments were size-fractionated on 0.75% agarose gel (SeaKem GTG; FMC BioProducts, Rockland, ME, USA) and blotted by alkali transfer with 4 M NaOH onto a positively charged nylon membrane (Biodyne Plus; Pall Corp., Pensacola, FL, USA). As a probe, we used the PCR product (about 0.8 kb) containing part of the *hph* gene. Probe labeling, hybridization, and detection were all done according to the manufacturer's protocols (AlkPhos Direct Labeling and Detection System with CDP-Star; GE Healthcare, Little Chalfont, UK; LAS1000 Luminescent Image Analyser; Fuji Photo Film Co. Ltd., Tokyo, Japan).

The result of transformation experiments is summarized in Table 1 and Fig. 1. Transformation experiments were performed with *A. tumefaciens* EHA105 containing the pPZP-HYG2 plasmid (Walton et al. 2005). Three mushroom species, *H. marmoreus*, *F. velutipes* and *G. frondosa*, were treated and transformed. *H. marmoreus* showed high transforming frequency: 86 hygromycin-resistant strains were obtained from 17 filters. Four resistant strains of *F. velutipes* and 2 resistant strains of *G. frondosa* were obtained (Table 1).

To test the sensitivities of transformants to hygromycin B, we grew the strains on medium containing hygromycin B and then measured colony growth (Fig. 1). The resistant strains could grow on medium containing 100 μg/ml hygromycin B, whereas the wild types did not grow. Wild-type colonies of *H. marmoreus* and *F. velutipes* would not grow in the presence of hygromycin B at concentrations of 25 μg/ml or higher. Wild-type *G. frondosa* would not grow on medium containing hygromycin B at 50 μg/ml or 100 μg/ml.

To confirm the insertion of the selectable marker gene into the transformant genomes, we performed PCR using primers for the *hph* gene and the *hph* fused actin promoter from *C. neoformans* in wild-type and transformant strains.

Four randomly selected transformants of *H. marmoreus*, all 4 transformants of *F. velutipes*, and all 2 transformant strains of *G. frondosa* were used for both the experiments. PCR analysis to detect transformants gave the expected bands, which were not detected in DNA from the untransformed mycelia of wild-type strains. Every resistant strain tested gave obvious bands [Fig. 2 and Supplemental Fig. (a) in electronic

Table 1 – Results of *Agrobacterium*-mediated transformation.

Species	No. of filter used	No. of obtained hygromycin B resistant colonies
Hypsizigus marmoreus	17	86
Flammulina velutipes	21	4
Grifola frondosa	18	2