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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.

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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

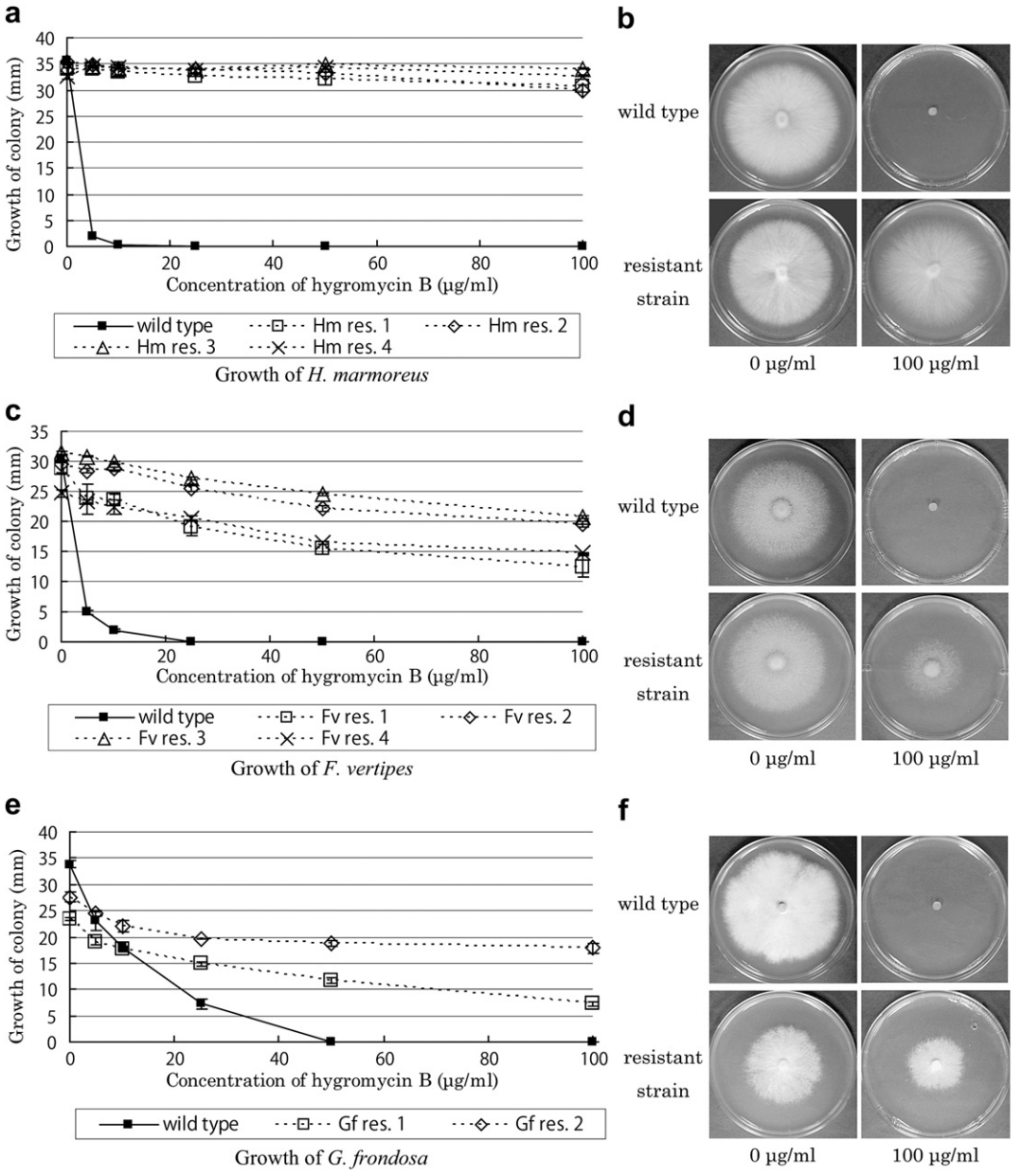

Fig. 1 – Growth of transformants and wild types on medium containing hygromycin B. a. Radii of *Hypsizigus marmoreus* colonies 11 d after inoculation. b. Photographs of *H. marmoreus* colonies 11 d after inoculation. c. Radii of *Flammulina velutipes* colonies 6 d after inoculation. d. Photographs of *F. velutipes* colonies 6 d after inoculation. e. Radii of *Grifola frondosa* colonies 22 d after inoculation. f. Photographs of *G. frondosa* colonies 22 d after inoculation.

supplementary materials]. These results confirmed that actin promoter of *C. neoformans* and *hph* gene had been introduced into the resistant strains.

Four transformants of *H. marmoreus*, three of *F. velutipes*, and two of *G. frondosa* were also subjected to Southern blot analysis. The transformants yielded bands of different sizes, whereas the wild-type strains yielded no signal. This indicated that *hph* gene cassette had been randomly inserted into the genomes of the mushroom species [Supplemental Fig. (b–e) in electronic supplementary materials].

We showed here that 3 mushroom species could be transformed by using the *Agrobacterium*-mediated method. Details of the transformation of *H. marmoreus* and *G. frondosa* have not been published previously. While this paper was in preparation, Okamoto et al. (2010) reported that mycelia of *F. velutipes* can be transformed by AMT. However, in our study the *hph* marker gene was expressed under the control of the actin promoter of *C. neoformans*, whereas Okamoto et al. used the native promoter of glyceraldehyde-3-phosphate dehydrogenase gene (*GPD*). Hygromycin B dose testing indicated that *hph* expression in the transformants of all 3 mushroom species genes was sufficient. Thus the actin promoter of *C. neoformans* functioned adequately in 4 distantly related species—*F. velutipes*, *H. marmoreus*, *G. frondosa*, and

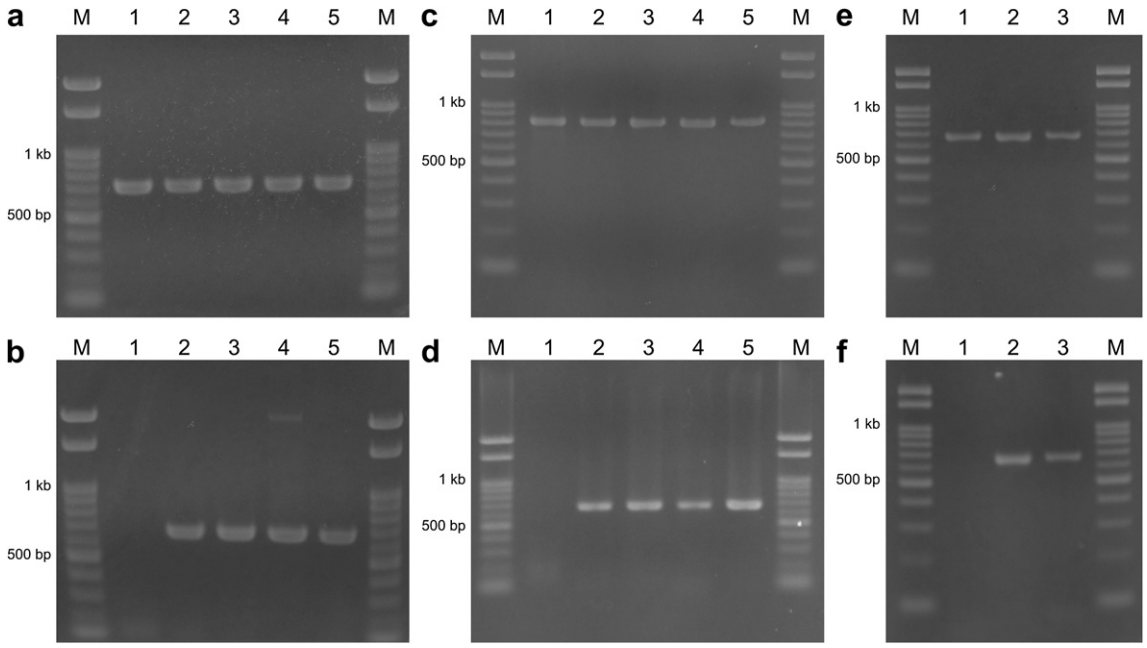

Fig. 2 – PCR analysis of genomic DNA prepared from independent transformants and wild types. a. PCR targeted to the fungal ribosomal ITS of *Hypsizigus marmoreus* genome. *M* size markers of 100 bp ladder, *1* untransformed wild type, *2–5* individual transformants. b. PCR targeted to the *hph* gene of *H. marmoreus* genome. *M* size markers of 100 bp ladder, *1* untransformed wild type, *2–5* individual transformants. c. PCR targeted to the fungal ribosomal ITS of *Flammulina velutipes* genome. *M* size markers of 100 bp ladder, *1* untransformed wild type, *2–5* individual transformants. d. PCR targeted to the *hph* gene of *F. velutipes* genome. *M* size markers of 100 bp ladder, *1* untransformed wild type, *2–5* individual transformants. e. PCR targeted to the fungal ribosomal ITS of *Grifola frondosa* genome. *M* size markers of 100 bp ladder, *1* untransformed wild type, *2 and 3* individual transformants. f. PCR targeted to the *hph* gene of *G. frondosa* genome. *M* size markers of 100 bp ladder, *1* untransformed wild type, *2 and 3* individual transformants.

C. neoformans—implying that the promoter is likely able to function in most basidiomycetous fungi. For ascomycetous fungi, the *trpC* promoter of *A. nidulans* is known as a universal promoter. For AMT of mushroom species, various promoters have been used to control the selectable markers. The *GPD* promoters of *A. bisporus* have often been used to drive the expression of marker genes in a wide range of mushrooms (Chen et al. 2000; Hanif et al. 2002; Combier et al. 2003; Godio et al. 2004; Cho et al. 2006; Ding et al. 2011). The *Schizophyllum commune GPD* promoter also drives the expression of genes in 3 different mushrooms (Pardo et al. 2002). The *GPD* promoters of *A. bisporus* or *S. commune* can be used as universal promoters for mushrooms. Our result suggests that the actin promoter of *C. neoformans* is the third potent candidate for use as a valuable universal promoter in basidiomycetes.

The signals of Southern blot hybridization indicated that the T-DNAs were inserted at random sites on the genomes of all three mushrooms, as previously shown in various fungi. The results in *H. marmoreus* and *G. frondosa* were similar to those of previous studies (Fitzgerald et al. 2003; Michielse et al. 2005; Sugui et al. 2005) in that the T-DNA was frequently inserted as a single copy, although one of the *F. velutipes* transformants had a double copy of T-DNA in its genome. This result suggests that AMT can be a useful tool for random insertional mutagenesis of *H. marmoreus* and *G. frondosa*. This efficient insertional mutagenesis by AMT, called T-DNA tagging, has been used to find new genes in plants and fungi (Koncz et al. 1990; Idnurm et al. 2004; Walton et al. 2005; Weld et al. 2006).

Use of the AMT system described here will enhance mushroom research, including exploration of genes of interest by T-DNA tagging or expression of useful proteins. The fact that all of the fungi investigated here were mushrooms should help to ensure progress in studies of fruiting in fungi.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.myc.2012.08.002.

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