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Agrobacterium-mediated Transformation of the Winter Mushroom, *Flammulina velutipes*

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Flammulina velutipes **was transformed efficiently by** ***Agrobacterium*****-mediated transformation system. The transformation frequency was about 16% with the gill tissues of the fungal fruiting body. Southern hybridization and genetic analysis suggest that the introduced DNA was inserted onto different locations of the fungal genome, and inherited stably to the next generation via basidiospores. Transformation or gene tagging with** ***Agrobacterium*** **T-DNA based vector should be useful for wide ranges of genetic or molecular biological studies of the mushroom.**

KEYWORDS: ***Agrobacterium, Flammulina velutipes,*** **Gene tagging, Marker inheritance, Transformation**

Winter mushroom, *Flammulina velutipes* is widely cultivated in Japan (Magae *et al.*, 2005) and Korea (Yoo *et al.*, 2005). This mushroom has been studied not only for practical application but also for basic research because it has been used as a model organism of fungal graviresponse (Kern *et al.*, 1997; Moore and Stockus, 1998). To solve many biological questions, molecular techniques have been used. Among the techniques, transformation and gene tagging are essential tools for the studies of molecular genetics, molecular breeding, and functional genomics in economically important mushrooms. Most of transformation methods for mushrooms were based on electroporation of protoplasts (Van de Rhee *et al.*, 1996), treatment with $CaCl_2$ and polyethylene glycol (Kim *et al.*, 2003; Ogawa *et al.*, 1998), or restriction enzyme-mediated integration (REMI, Joh *et al.*, 2003; Hirano *et al.*, 2000; Irie *et al.*, 2003). *Agrobacterium*-mediated transformation (AMT) has some advantages over the other methods. One of them is that it does not need to prepare the protoplasts. Since it had been reported that T-DNA from *Agrobacterium tumefaciens* could transform *Saccharomyces cerevisiae* (Bundock *et al.*, 1995) and some filamentous fungi (de Groot *et al.*, 1998), *Agrobacterium*-mediated transformation system was developed for many important fungi including *Agaricus bisporus* (Rhee, 1996; Chen *et al.*, 2000; Mikosch *et al.*, 2001). In this study, we transformed economically important mushroom, *Flammulina velutipes* by AMT which is the first report in *F. velutipes.*

Transformation of *F. velutipes* was carried out with the *Agrobacterium tumefaciens* AGL-1 strain containing pBGgHg (Chen *et al.*, 2000). The plasmid has hygromycin B resistance gene (*hph*, hygromycin B phosphotranferase) as a selection marker with the *gpd* (glyceraldehydes-3-phosphate dehydrogenase) promoter from *A. bisporus* and the CaMV 35S terminator. The *Agrobacterium* was grown in minimal medium (MM salts-K_2HPO_4 2.05 g, KH_2PO_4 1.45 g, NaCl 0.15 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCl_2 \cdot 6H_2O$ 0.1 g, $FeSO_4 \cdot 7H_2O$ 2.5 mg, $(NH_4)_2SO_4$ 0.5 g, and 2 g per liter; Hooykaas *et al.*, 1979) having kanamycin at 50 μg/ml for 2 days at 25 using a rotary shaker set with 200 rpm. The culture was diluted to an optical density of 0.15 at 600 nm in the induction medium (MM salts containing 40 mM MES pH 5.3, 10 mM glucose, 0.5% glycerol, and 200 μM acetosyringone; Bundock *et al.*, 1995), and further cultured for 6 hr. The gill tissues of *Flammulina velutipes* fruiting body were aseptically excised and sectioned into 2 to 3-mm square pieces. The tissue pieces were vacuum infiltrated for 10 minutes with the suspension of the bacteria grown in induction medium, and was transferred to co-cultivation agar medium (induction medium containing 5 mM glucose instead of 10 mM glucose). After incubation on the co-cultivation agar for 3 days at 25°C~28°C, the tissue pieces were transferred to selection agar medium (malt extract agar containing 50 μg/ml hygromycin, 200 μM cefotaxime and 100 μg/ml moxalactum). The mycelium grown from the gill tissue piece on the selection agar medium was transferred to new selection agar medium, and finally to malt extract agar medium containing 50 μg/ml hygromycin.

The hygromycin resistant transformants were cultured in potato dextrose broth (Becton, Dickinson & Co., Sparks, MD, USA) for 30~45 days, and mycelial mats were harvested from the cultures and freeze-dried. Total DNA of the freeze dried mycelium was isolated with a

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Genomic DNA Isolation Kit (NucleoGen, Siheung, Gyeonggi, Korea) or with the method described by Ishii *et al.* (2001). PCR was conducted using primers, gpd-FH (5'GAAGAAGCTTTAAGAGGTCCGC3') and hph-R (5' GGCGACCTCGTATTGGGAATC3') (Chen *et al.*, 2000).

For Southern hybridization analysis, 10 μg of total DNA was restricted with *Sac*I, separated on a 0.7% agarose gel, and transferred to a nylon membrane by capillary blotting. Hybridization and detection were carried out with Dig High Prime DNA Labelling and Detection Kit II (Roche Diagnostics GmbH, Manneheim, Germany). The DNA amplified by PCR with gpd-FH and hph-R was used as a probe. Fruiting body of hygromycin resistant transformants (T0 generation) was obtained by conventional cultivation method of winter mushroom. Single spore cultures (haploid, T1 generation) were obtained from the T0 fruiting body and maintained on PDA. Hygromycin resistance of the T1 single spore culture was determined on MEA containing hygromycin (50 μg/ml).

It was observed that some hypha grew on the selection agar out of the sixteen gill tissues among 100 gill tissues treated with the *A. tumefaciens* AGL-1. PCR with the primers specific for hygromycin resistance gene amplified the expected size of DNA from all 16 hygromycin resistant transformants, but it did not form the hygromycin sensitive transformants (Fig. 1). Southern hybridization using the probe of the hygromycin resistance gene showed the hybridized band on all hygromycin resistant transformants (Fig. 3). These results suggest that the 16 hygromycin resistant transformants out of 100 tissues were transformed with the marker, hygromycin resistant gene from *A. tumefaciens* AGL-1. Transformation frequency, 16%, is comparable to transformation frequency of *Agaricus bisporus* in Chen *et al*'s study (Chen *et al.*, 2000) in which the gill tissue of *A. bisporus* was used for transformation and the frequency was 30~40%. The sizes of the hybridized bands detected by Southern hybridization were different one another, which suggests the marker genes were inserted onto different locations of the *F. velutipes* genome. Kue *et al.* (2004) showed the hygromycin resistant marker gene inserted once in different locations of *F. velutipes* genome by non-homologous recombination in their transformation study by electroporation of basiospores.

The 100 T1 generation single spore cultures from each 16 hygromycin resistant transformants were segregated in their response to hyg B with the ratio of 1 resistance and 1 sensitive except (Table 1). Hygromycin resistant gene

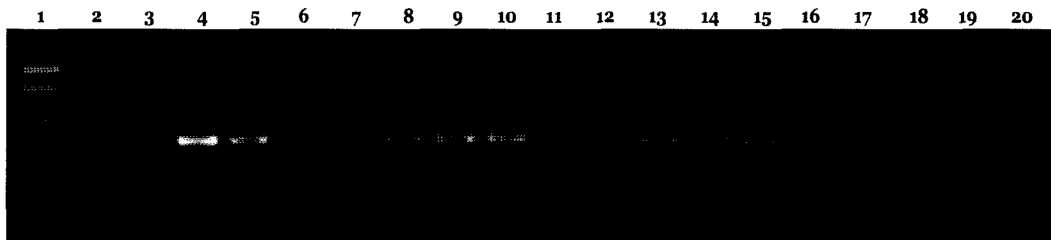

Fig. 1. PCR analysis of DNA isolated from the hygromycin resistant transformants (T0) of *F. velutipes*. PCR amplification was carried out using primers, gpd-FH and hph-R, defining a 970 bp sequence spanning the *gpd* promoter and the *hph* gene. Lanes 2~3 for DNA from negative control and lane 4 for pBGgHg as a positive control and lanes 5~20 for DNA from 1~16 hygromycin resistant transformants.

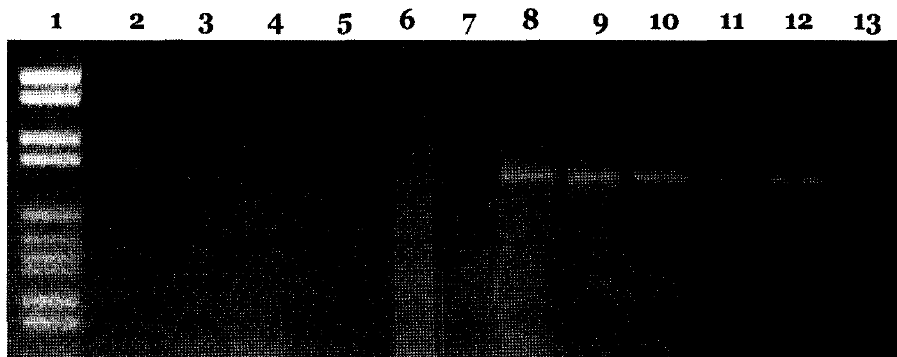

Fig. 2. PCR analysis of DNA isolated from the single spore cultures (T1) from the hygromycin resistant transformants (T0) of *F. velutipes*. PCR amplification was carried out using primers, gpd-FH and hph-R, defining a 970 bp sequence spanning the *gpd* promoter and the *hph* gene. Lanes 2~7 for DNA from hygromycin sensitive single spore cultures and lanes 8~13 for DNA from hygromycin resistant single spore cultures.

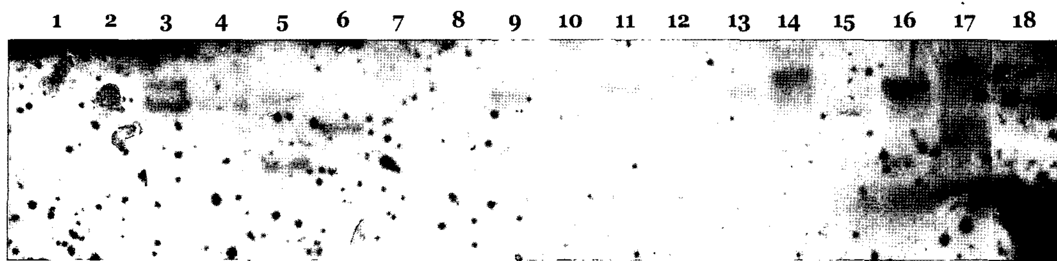

Fig. 3. Southern blot hybridization analysis of *F. velutipes* transformants. Genomic DNA from the transformants (T0) was digested with *SacI* and probed with the *hph* gene. Lane: 1 hygromycin sensitive strain as negative control, lanes 2~17: hygromycin resistant transformants, and lane 18: *hph* gene probe.

Table 1. Segregation ratio of the hygromycin resistant gene in T1 generation single spore cultures

Transformant (T0)	Single spore cultures (T1)		χ^2 test
	Hyg^R	Hyg^S	
T0-1	54	46	0.64
T0-2	50	50	0
T0-3	47	53	0.36
T0-4	54	46	0.64
T0-5	58	42	2.56
T0-6	59	41	3.24
T0-7	55	45	1.0
T0-8	53	47	0.36
T0-9	54	46	0.64
T0-10	60	40	4.0*
T0-11	57	43	1.96
T0-12	56	44	1.44
T0-13	50	50	0
T0-14	58	42	2.56
T0-15	52	48	0.16
T0-16	49	51	0.04

* Indicates significantly different (0.05 < P < 0.025).

specific DNA was amplified only in hyg^R T1 clones (Fig. 2). The Southern hybridization also showed the hybridized band only in the hyg^R T1 clones (data not shown). These results indicate that the marker gene was inserted once and the gene was maintained stably during meiosis and inherited to the next generation via basidiospore. This is the first result showing the transformed foreign gene inherited to the next generation through meiosis in *F. velutipes*. Kue *et al.* (2004) confirmed the marker gene that is the same gene as used in this study maintained stably during mitotic cell division for 3 months in mycelium.

Transformation of *F. velutipes* by AMT showed in this study is easy to carry out with moderate efficiency and the marker gene was inserted once in the fungal genome randomly and inherited to the next generation through meiosis. This AMT method should be usefully applied for the molecular genetic analysis, molecular breeding, and biotechnological application of the economically important winter mushroom.

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