Mycoscience (2006) 47:228–231
DOI 10.1007/s10267-006-0293-9

SHORT COMMUNICATION

Hitoshi Murata · Tomohiro Igasaki · Kazuo Shishido
Masahide Sunagawa

Agrobacterium-mediated transformation of the ectomycorrhizal basidiomycete *Tricholoma matsutake* that produces commercially valuable fruit bodies, matsutake

Received: October 18, 2005 / Accepted: March 27, 2006

Abstract Using agroinfection with a T-DNA vector carrying a hygromycin resistance marker, the recombinants were generated for the first time from the ectomycorrhizal basidiomycete *Tricholoma matsutake*, which produces commercially valuable fruit bodies, matsutake, during association with *Pinus* sp. plants. The transformation system may be useful in the genetic analysis of *T. matsutake*.

Key words *Agrobacterium* · Ectomycorrhizal fungi · Transformation; *Tricholoma matsutake*

Tricholoma matsutake is an ectomycorrhizal basidiomycete that produces economically important edible mushrooms, matsutake, in association with *Pinus* sp. plants in the Northern Hemisphere (Hosford et al. 1997). Although the ecology of *T. matsutake* has been elucidated, the analysis of biological function based on molecular genetics has been hampered by technical barriers in this symbiont (Guerin-Lagette et al. 2002, 2004; Hosford et al. 1997; Murata et al. 2005; Yamada et al. 1999). A major constraint in such an analysis is the lack of a transformation system in *T. matsutake*, in contrast to other basidiomycetes. In fact, many transformation systems developed so far in basidiomycetes were for wood-rotting basidiomycetes and the common mushroom, whereas only a few cases have been reported in ectomycorrhizal basidiomycetes (Burns et al. 2005; Chen et al. 2000; Hanif et al. 2002; Ma et al. 2001; Marmeisse et al. 1992; Ogawa et al. 1998; Orihara et al. 2005). Such a method, if developed, will facilitate molecular genetic analysis of *T. matsutake* to elucidate the biological functions involved in symbiosis, the unique colonization in the rhizosphere forming a massive aggregate called *shiro*, and fruit-body production, information that may be useful in the development of artificial culture practice. In the present study, we developed an *Agrobacterium*-mediated transformation system to generate recombinants of *T. matsutake*.

The T-DNA vector pHHM203 was constructed based on the plasmid pIGA0200 and pLC1-hph (Igasaki et al. 2000; Ogawa et al. 1998). pIGA0200 is derived from pSMAH704, which was designed to transform the plant *Robinia pseudoacacia* (Igasaki et al. 2000). pIGA0200 contains *ori*$^+$-ColE1 required for the replication in *Escherichia coli* and *ori*$^+$-pVS1 for the replication in *Agrobacterium tumefaciens*, spectinomycin resistance marker (Spc^r), *sta*$^+$ required for plasmid stability, and a multiple cloning site (*Bgl*II, *Pst*I, *Hin*dIII, *Sal*I, *Xba*I, *Kpn*I, *Eco*RI) within the T-DNA region. pLC1-hph is a pUC19 derivative containing hygromycin B resistance marker (Hyg^r), which consists of a promoter region of the *ras* gene from the wood-rotting basidiomycete *Lentinula edodes* (P*ras*), hygromycin phosphotransferase gene from *E. coli* (*hph*), and a terminator region of the *priA* gene from *L. edodes* (T*priA*) (Ogawa et al. 1998). The *Eco*RV-*Mun*I fragments containing P*ras*-*hph*-T*priA* of pLC1-hph and the *Hin*dIII-*Xba*I digests of pIGA0200 containing all the elements required for agroinfection were blunt-ended by KOD DNA polymerase reaction (Toyobo, Osaka, Japan), and ligated together to generate the T-DNA vector plasimd pHHM203 (Fig. 1). pHHM203 was introduced into *A. tumefaciens* strains AGL-1 and EHA105, which were provided by Mark Guiltinan at the Pennsylvania State University through electroporation.

A method described by Hanif et al. (2002) was modified to transform *T. matsutake* Y1, Y4, and F1, the strains widely used in scientific research as a model of the species (Yamada et al. 1999). *Agrobacterium tumefaciens* carrying the vector plasmid was precultured on minimal salts–0.2%

H. Murata (✉) · M. Sunagawa
Department of Applied Microbiology and Mushroom Science, Forestry & Forest Products Research Institute, 1 Matsunosato, Tsukuba 305-8687, Japan
Tel. +81-298-73-3211; Fax +81-298-73-0507
e-mail: murmur@ffpri.affrc.go.jp

T. Igasaki
Department of Molecular Cell Biology, Forestry & Forest Products Research Institute, Tsukuba, Japan

K. Shishido
Department of Life Science, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Kanagawa, Japan

glucose (SGc) agar containing 100 µg/ml spectinomycin (Spc; Sigma, St. Louis, MO, USA) at 28°C (Murata et al. 1991). Then, bacterial cells were inoculated into a SGc liquid medium containing Spc and 200 µM 4-hydroxy-3,5-dimethoxy acetophenone (acetosyringone or Acs; Aldrich Japan, Tokyo, Japan), and grown in a 28°C rotary shaker to the midexponential growth phase ($OD_{600} \cong 0.9$) to be used for transformation. *Tricholoma matsutake* mycelia were grown to the diameter of ~ 1 cm on a nylon membrane (1.5 × 1.5 cm, pore size = 20 µm; e.g., Hybond-N; Amersham Pharmacia Biotech, Buckinghamshire, UK) overlaid on the modified MMN containing 1.5% V8 juice (Campbell Soup, Camden, NJ, USA) agar at 25°C (MMN-V8; Murata et al. 1999). Mycelia on the membrane were dipped in the culture of *A. tumefaciens* carrying a vector for 10 min and then placed on MMN-V8 agar containing Acs. After incubation at 25°C for 6 days on the agar plate containing Acs, mycelia on the membrane were washed once in sterile 100 µg/ml cefotaxime (Cef; Wako Pure Chemical, Osaka, Japan) solution, and placed on MMN-V8 containing 100 µg/ml hygromycin B (Hyg; Wako Pure Chemical) and Cef, and incubated at 25°C. As a control, *T. matsutake* mycelia were treated with *A. tumefaciens* AGL-1 and EHA105 without the plasmid in the same way as done for agroinfection. Six replicates were made for each agroinfection with *A. tumefaciens* AGL-1 carrying pHHM203 (= AGL-1/pHHM203), EHA105 carrying pHHM203 (= EHA105/pHHM203), AGL-1, or EHA105. Transformation efficiency was expressed as the percentage of replicates having

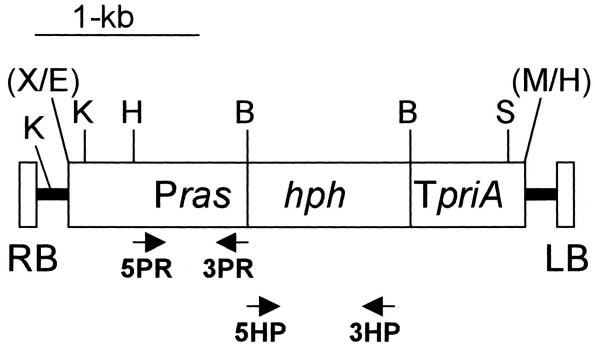

Fig. 1. Schematic representation of the T-DNA region of pHHM203. *Open boxes* indicate features of the T-DNA region including LB (*left border*) and RB (*right border*). *Solid lines* depict multiple cloning sites of pIGA0200. Relevant restriction sites are given: *B*, *Bam*HI; *E*, *Eco*RV; *H*, *Hin*dIII; *K*, *Kpn*I; *M*, *Mun*I; *S*, *Sac*I; *X*, *Xba*I; (/), the blunt-end ligation site after flashing the protruding ends with KOD DNA polymerase. *Arrows* indicate primers for polymerase chain reaction (PCR) analysis

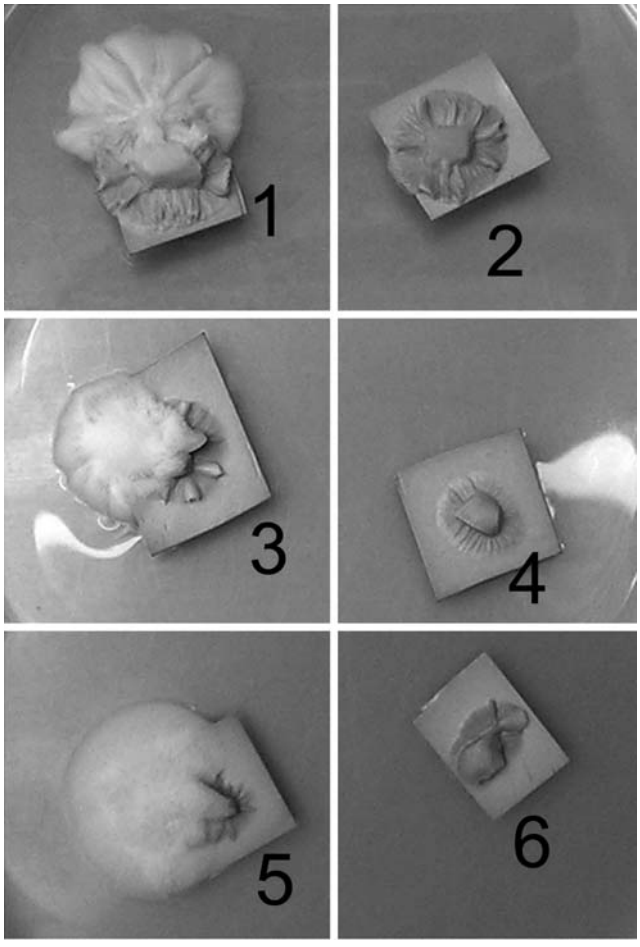

Fig. 2. Expression of the Hyg^r trait in *Tricholoma matsutake*. *1*, *T. matsutake* Y1 carrying pHHM203 after agroinfection; *2*, *T. matsutake* Y1 dipped in the culture of *Agrobacterium tumefaciens* without the plasmid; *3*, *T. matsutake* Y4 carrying pHHM203 after agroinfection; *4*, *T. matsutake* Y4 dipped in the culture of *A. tumefaciens* without the plasmid;. *5*, *T. matsutake* F1 carrying pHHM203 after agroinfection; *6*, *T. matsutake* F1 dipped in the culture of *A. tumefaciens* without the plasmid. Please note that fresh white mycelia are growing from darkened inocula after agroinfection with pHHM203. Photograph was taken 46 days after treating with *A. tumefaciens*

Table 1. Occurrence of Hyg^r recombinants from *Tricholoma matsutake* through agroinfection

T. matsutake strain	Number of replicates showing Hyg^r recombinants[a]		Transformation efficiency[b] (%)
	Agrobacterium tumefaciens strain (plasmid)		
	AGL1 (pHHM203)	EHA105 (pHHM203)	
Y1	3	3	50
Y4	4	4	67
F1	6	4	83

[a] Six replicates were made for each agroinfection; no growth was noted in any *T. matsutake* mycelia treated with *A. tumefaciens* without the vector plasmid

[b] Transformation efficiency (%) was expressed as the percentage of replicates showing Hyg^r recombinants relative to total replicates of each fungal specimen tested for agroinfection

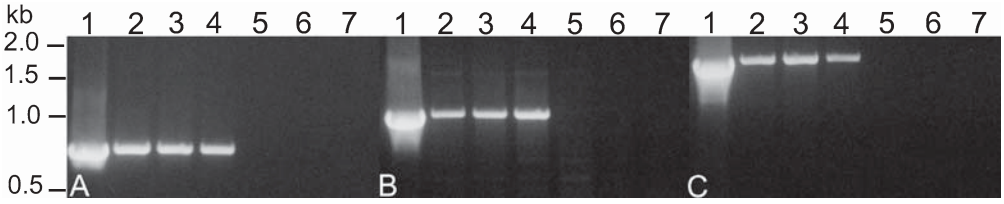

Fig. 3. PCR analysis of total DNA prepared from recombinants and wild types. *A*, PCR targeting P*ras* (= 0.7 kb); *B*, PCR targeting *hph* (= 1.1 kb); *C*, PCR targeting P*ras-hph* (= 1.7 kb). *Lane 1*, pHHM203; *2*, *T. matsutake* Y1 carrying pHHM203; *3*, *T. matsutake* Y4 carrying pHHM203; *4*, *T. matsutake* F1 carrying pHHM203; *5*, *T. matsutake* Y1; *6*, *T. matsutake* Y4; *7*, *T. matsutake* F1. Molecular markers (kb) are given

produced Hyg^r recombinants relative to total replicates of each fungal specimen tested for agroinfection.

Hyg^r recombinants were generated from *T. matsutake* Y1, Y4, and F1 at frequencies ranging from 50% to 83% depending upon fungal strains (Table 1, Fig. 2). Both *A. tumefaciens* AGL-1 and EHA105 were capable of transferring the T-DNA vectors to *T. matsutake* to the same extent (Table 1). No growth was noted in any *T. matsutake* mycelia dipped in the culture of *A. tumefaciens* without the vector plasmid (Fig. 2). All the recombinants exhibited the Hyg^r phenotype even after repeated subculturing during a 6-month period.

To determine the presence of P*ras-hph*-T*priA* in Hyg^r strains, total DNA was extracted from the recombinants transferred several times on the selection agar plate during a 6-month period and from the wild types by a standard protocol using a lysis buffer containing hexadecyltrimethylammonium bromide and phenol-chloroform (Dobinson et al. 1993). Then, total DNA was analyzed by polymerase chain reaction (PCR) with a set of primers targeting P*ras* [5PR = 5′-CCCTTCGCATAGCGGGAT CATATGAATC-3′/3PR = 5′-GATTCAGAAACAGTCG GCTCCTCAAGTC-3′ (T_m = 63°C)], with those targeting *hph* [5HP = 5′-GAGATATGAAAAAGCCTGAACTC ACCGC-3′/3HP = 5′-CGGTTTCCACTATCGGCGAG TACTTCT-3′ (T_m = 63°C)], and with those targeting P*ras-hph* (5PR/3HP) (Fig. 1; Ogawa et al. 1998). Cycle reactions were performed as follows; 1× (94°C/2 min), 30× (94°C/30 s, 63°C/30 s, 72°C/2 min), 1× (72°C/10 min). PCR products were analyzed by TAE-1% agarose gel electrophoresis. The analysis identified the 1.0-kb, 0.7-kb, and 1.7-kb DNA segments that correspond to *hph*, P*ras*, and P*ras-hph* respectively, in the Hyg^r recombinants of *T. matsutake* as detected in pHHM203, whereas no such DNA segments were amplified in the samples of the wild types (Fig. 3).

This is the first report of *Agrobacterium*-mediated transformation of *T. matsutake*. In the course of the analysis, we found that *T. matsutake* mycelia grew horizontally on a nylon membrane overlaid on the MMN-V8 agar faster than on the agar plate without the nylon membrane. Therefore, the protocol described here allows the transformation of *T. matsutake* to be simple and highly reliable. Considering the fact that *T. matsutake* hardly ever confers a monokaryon, even through spore germination, unlike many other homobasidiomycetes (A. Ohta and A. Yamada, personal communication), the transformation system may be more effective in functional analysis through the expression of cloned genes than in analysis through insertion mutagenesis. It is interesting to test how *T. matsutake* behaves when genes involved in biodegradation from wood-rotting basidiomycetes were introduced. By introducing autofluorescent protein expression systems into *T. matsutake*, we may be able to prove experimentally heterologous mycelial interactions during the formation of *shiro* (Babasaki et al. 2003; Murata et al. 2005; Yamada et al. 2006).

To carry out such an analysis, we are currently developing a binary vector containing the Hyg^r marker and a cassette that allows the expression of a given gene by using two different promoters and terminators to drive *hph* and a gene of interest, respectively. Please note that the *Hin*dIII and *Kpn*I sites outside of P*ras* and the *Sac*I site outside of T*priA* can be used in inserting another expression cassette (see Fig. 1). Once a binary vector is developed, genetic analysis of the ectomycorrhizal symbiosis will significantly advance. The *Agrobacterium*-mediated transformation system described here is, however, a great step toward genetic analysis of commercially valuable but yet uncultivable matsutake.

Acknowledgment We are grateful to Dr. Mark Guiltinan, Pennsylvania State University, for provision of *Agrobacterium tumefaciens* strains. This work was supported by a grant from the Ministry of Agriculture, Forestry and Fishery of Japan.

References

Babasaki K, Masuno K, Murata H (2003) Interactions of heterologous mycelia colonized in the substrate govern fruit body production in the cultivated homobasidiomycete *Pholiota nameko*. Biosci Biotechnol Biochem 67:100–106

Burns C, Gregory KE, Kirby M, Cheung MK, Riquelme M, Elliott TJ, Challen MP, Bailey A, Foster GD (2005) Efficient GFP expression in the mushrooms *Agaricus bisporus* and *Coprinus cinereus* requires introns. Funct Genet Biol 42:191–199

Chen X, Stone M, Schlagnhaufer C, Romaine CP (2000) A fruit body tissue method for efficient *Agrobacterium*-mediated transformation of *Agaricus bisporus*. Appl Environ Microbiol 66:4510–4513

Dobinson KF, Harris RE, Hamer JE (1993) *Grasshopper*, a long terminal repeat (LTR) retroelement in the phytopathogenic fungus *Magnaporthe grisea*. Mol Plant-Microbe Interact 6:114–126

Guerin-Lagette A, Matsushita N, Kikuchi K, Iwase K, Lapeyrie F, Suzuki K (2002) Identification of a prevalent *Tricholoma matsutake* ribotype in Japan by rDNA IGS spacer characterization. Mycol Res 106:435–443

Guerin-Lagette A, Shindo K, Matsushita N, Suzuki K, Lapeyrie F (2004) Mycorrhizal fungus *Tricholoma matsutake* stimulates *Pinus densiflora* seedling growth *in vitro*. Mycorrhiza 14:397–400

Hanif M, Pardo AG, Gorfer M, Raudaskoski M (2002) T-DNA transfer and integration in the ectomycorrhizal fungus *Suillus bovinus* using hygromycin B as a selectable marker. Curr Genet 41:183–188

Hosford D, Pilz D, Molina R, Amaranthus M (1997) Ecology and management of the commercially harvested American matsutake mushroom. USDA Forest Service, PNW-GTR412, Portland, pp 1–68

Igasaki T, Mohri T, Ichikawa H, Shinohara K (2000) *Agrobacterium tumefaciens*-mediated transformation of *Robinia pseudoacacia*. Plant Cell Rep 19:448–453

Ma B, Mayfield MB, Gold MH (2001) The green fluorescent protein gene functions as a reporter of gene expression in *Phanerochaete chrysosporium*. Appl Environ Microbiol 67:948–955

Marmeisse R, Gay G, Debaud JC, Casselton LA (1992) Genetic transformation of the symbiotic basidiomycete fungus *Hebeloma cylindrosporum*. Curr Genet 22:41–45

Murata H, McEvoy JL, Chatterjee A, Collmer A, Chatterjee AK (1991) Molecular cloning of an *aepA* gene that activates production of extracellular pectolytic, cellulolytic, and proteolytic enzymes in *Erwinia carotovora* subsp. *carotovora*. Mol Plant-Microbe Interact 4:239–246

Murata H, Yamada A, Babasaki K (1999) Identification of repetitive sequences containing motifs of retrotransposons in the ectomycorrhizal basidiomycete *Tricholoma matsutake*. Mycologia 91:766–775

Murata H, Ohta A, Yamada A, Narimatsu M, Futamura N (2005) Genetic mosaics in the massive persisting rhizosphere colony "shiro" of the ectomycorrhizal basidiomycete *Tricholoma matsutake*. Mycorrhiza 15:505–512

Ogawa K, Yamazaki T, Hasebe T, Kajiwara S, Watanabe A, Asada Y, Shishido K (1998) Molecular breeding of the basidiomycete *Coprinus cinereus* strains with high lignin-decolorization and - degradation activities using novel heterologous protein expression vectors. Appl Microbiol Biotechnol 49:285–289

Orihara K, Yamazaki T, Shinkyo R, Sakai T, Inouye K, Tsukamoto A, Sugiura J, Shishido K (2005) Rat cytochrome P450-mediated transformation of dichlorodibenzo-*p*-dioxins by recombinant white-rot basidiomycete *Coriolus hirsutus*. Appl Microbiol Biotechnol 69:22–28

Yamada A, Maeda K, Ohmasa M (1999) Ectomycorrhiza formation of *Tricholoma matsutake* isolates on seedlings of *Pinus densiflora* in vitro. Mycoscience 40:455–463

Yamada A, Maeda K, Kobayashi H, Murata H (2006) Ectomycorrhizal symbiosis *in vitro* between *Tricholoma matsutake* and *Pinus densiflora* seedlings that resembles naturally occurring "shiro". Mycorrhiza 16:111–116