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ORIGINAL PAPER

# Expression of the autofluorescent protein, DsRed2, in the recombinants of the ectomycorrhizal basidiomycete, *Suillus grevillei*, generated by *Agrobacterium*-mediated transformation

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**Abstract** Recombinants were generated from the ectomycorrhizal basidiomycete, *Suillus grevillei*, through agro-infection using a binary vector carrying the hygromycin B resistance and the autofluorescent protein, DsRed2, markers. DsRed2 was driven by a *cis*-regulatory region of the glyceraldeyde-3-phosphate dehydrogenase gene (*gpd*) from the wood-rotting basidiomycete, *Coriolus hirsutus*, which contains promoters and 5′ *gpd* sequences with first through fourth exons and expressed for the first time in *Suillus* spp. The transformation system and recombinants expressing an autofluorescent protein may be useful in genetic analysis of the symbiosis.

**Keywords** *Agrobacterium* · Autofluorescent protein · DsRed2 · Ectomycorrhizal fungi · *Suillus grevillei*

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## Introduction

*Suillus* spp. are ectomycorrhizal basidiomycetes that associate with conifers especially in adverse environment worldwide, such as sand dunes, rocky mountains, volcanic areas, frigid regions, and chemically contaminated lands (Richardson 1998; Smith and Read 1997). The symbiosis brings about enhanced above-ground plant growth and development of mycorrhizal root systems underground (Smith and Read 1997). *Suillus grevillei* is a symbiont that associates with the larch, *Larix leptolepis*, a tree species that forms the largest boreal forest called taiga in Siberia (Smith and Read 1997). *L. leptolepis* generally grows faster than any other conifers during daylight. The symbionts can be easily cultured in an artificial medium and axenically infected to lateral roots of plant seedlings in vitro. Therefore, *Suillus* spp. are symbionts desirable for the analysis of physiology and biochemistry involved in host specificity and plant promoting mechanisms. They are also useful for preservation and restoration of forest lands (Richardson 1998; Smith and Read 1997). However, a genetic transformation system of *Suillus* spp. was not available until very recently.

Raudaskoski and her associates reported *Agrobacterium*-mediated genetic transformation of *Suillus bovinus*, which associates with the European red pine, *Pinus sylvestris*, with a vector carrying hygromycin B phosphotransferase (*hph*) and enhanced green fluorescent protein (*egfp*) genes (Hanif et al. 2002). In their system, both *hph* and *egfp* were driven by the promoter of glyceraldehyde-3-phosphate dehydrogenase (P*gpd*) from the common mushroom, *Agaricus bisporus*, and both terminated by CaMV *35s* terminator (T*35s*) (Chen et al. 2000). The recombinants, however, only expressed $Hyg^r$ but not EGFP (Hanif et al. 2002). Autofluorescent proteins, such as EGFP and

**Table 1** Plasmids

| Plasmids | Relevant characteristics | |
|---|---|---|
| pDsRed2 | Amp$^r$, DsRed2$^+$ | (Takara Clonetech, Otsu, Japan) |
| pIGA0621-EGFP | pSMAH704 derivative, Spc$^r$, *ori*$^+$ (ColE1), *ori*$^+$ (pVS1), *sta*$^+$, P*35s* from CaMV-*Ω* from TMV-*egfp*-T*nos* from *A. tumefaciens* | (Igasaki et al. 2000) |
| pIGA0200 | pSMAH704 derivative containing multiple cloning sites (*Bgl*II, *Pst*I, *Hin*dIII, *Sal*I, *Xba*I, *Kpn*I, *Eco*RI) within T-DNA, Spc$^r$ | (Igasaki et al. 2000) |
| pLC1-hph | Amp$^r$, P*ras* from *Lentinula edodes-hph* from *Eschrichia coli*-T*priA* from *L. edodes* | (Ogawa et al. 1998) |
| pMIp5 | Amp$^r$, P*gpd-5′gpd* containing sequences from 224 bp of 1st exon through 8 bp of 4th exon from *Coriolus hirsutus* | (Orihara et al. 2005) |
| pBluescript II SK+ | Amp$^r$ | (Stratagene, La Jolla, CA) |
| pUC18 | Amp$^r$ | (Toyobo, Osaka, Japan) |
| pHHM181 | pUC18 containing P*35s-Ω -egfp*-T*nos* at the *Hin*dIII-*Eco*RI sites (P*35s* at the *Hin*dIII-*Xba*I sites, *Ω -egfp* at the *Xba*I-*Sac*I sites, T*nos* at the *Sac*I-*Eco*RI sites) | |
| pHHM186 | pUC18 containing P*35s-dsred2*-T*nos* at the *Hin*dIII-*Eco*RI sites (*dsred2* at the *Xba*I-*Sac*I sites) | |
| pHHM188 | pUC18 containing P*gpd-5′ gpd- dsred2*-T*nos* at the *Hin*dIII-*Eco*RI sites (P*gpd-5′ gpd* at the *Hin*dIII-*Xba*I) | |
| pHHM190 | pBluescript II SK+ containing P*gpd-5′ gpd- dsred2*-T*nos* at the *Hin*dIII-*Eco*RI sites | |
| pHHM192 | pBluescript II SK+ containing P*gpd-5′ gpd- dsred2*-T*nos* and P*ras-hph*-T*priA* at the *Hin*dIII-*Eco*RI sites and the *Sma*I site, respectively | |
| pHHM194 | pIGA0200 containing P*gpd-5′ gpd- dsred2*-T*nos* and P*ras-hph*-T*priA* at the *Kpn*I site (Fig. 1) | |
| pHHM203 | pIGA0200 containing P*ras-hph*-T*priA* at the blunt-ended *Hin*dIII-*Xba*I sites | |

*Discosoma striata* red fluorescent protein (DsRed) or the enhanced derivative, DsRed2, allow us to monitor expression of given genes and behavior of given genets in situ in real time (Larrainzar et al. 2005). Therefore, autofluorescent proteins are useful in analyzing processes involved in various aspects of microbial phenomena, such as host–parasite interactions (Larrainzar et al. 2005).

Previously, we reported that the P*gpd-5′gpd* containing exons and introns from the wood-rotting basidiomycete, *Coriolus hirsutus*, promoted the expression of the rat cytochrome *P*450 gene in the homologous system (Orihara et al. 2005). In the present study, we report an *Agrobacterium*-mediated transformation system of *S. grevillei* with binary vectors. In this system, *dsred2* was driven by the P*gpd-5′gpd* from *C. hirsutus*, and the autofluorescent protein was expressed for the first time in *Suillus* spp.

## Materials and methods

### Strains and media

*S. grevillei* Sg-i3 was isolated from *L. leptolepis* forests in Iwate prefecture, Japan, in 2001. Fungal mycelia were cultured in modified Melin–Norkrans synthetic medium (MMN) modified by the addition of V8 juice (Campbell Soup, Camden, New Jersey) to the final concentration of 1.5% instead of NaCl (=MMN-V8) at 25°C. *Agrobacterium tumefaciens* AGL-1 and EHA105 were provided by Dr. Mark Guiltinan, the Department of Horticulture, the Pennsylvania State University. Unless stated otherwise, *A. tumefaciens* strains were cultured in minimal salts—20% glucose medium (SGc) at 28°C (Murata et al. 1991). *Escherichia coli* JM109 was obtained from Toyobo (Osaka, Japan). When required, the following antibiotics and the inducer of T-DNA transfer were incorporated into the media: 50-μg/ml ampicillin (Amp; Sigma, St Louis, MO), 100-μg/ml spectinomycin (Spc; Sigma), 100-μg/ml hygromycin B (Hyg; Wako Pure Chemical, Osaka, Japan), 100-μg/ml cefotaxime (Cef; Wako Pure Chemical), and 200-μM 4-hydroxy-3,5-dimethoxy acetophenone (=acetosyringone or Acs; Aldrich Japan, Tokyo).

### Molecular techniques

Standard procedures were used in transformation and electroporation of bacteria, polymerase chain reaction (PCR), gel electrophoresis, and restriction digests, dephosphorylation and ligation of DNA (Sambrook and Russell 2001). Isolation of plasmids from bacteria was carried out with the Wizard *Plus* SV minipreps DNA purification system (Promega, Madison Wisconsin) and QIAfilter Plasmid Maxi Kit (QIAGEN KK, Tokyo, Japan). DNA fragments were eluted from an agarose gel using Wizard

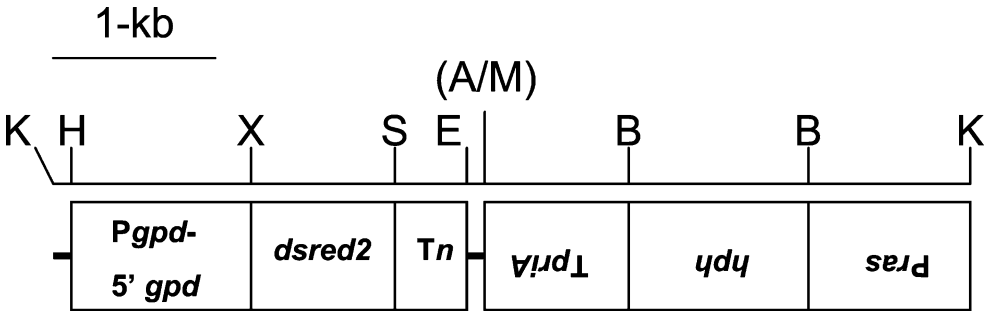


**Fig. 1** Schematic representation of the *hph* and *dsred2* expression cassettes. *Solid lines* indicate portions of pBluescript II SK+. Abbreviations: *B Bam*HI, *E Eco*RI, *H Hin*dIII, *K Kpn*I, *M Mun*I, *S Sac*I, *X Xba*I, *(A/M)* the blunt-end ligation site with *Sma*I and KOD-treated *Mun*I, *Tn* T*nos*

DNA purification system (Promega) after the separation in gel electrophoresis in TAE buffer. Genomic DNA was isolated from fungal mycelia using a lysis buffer containing hexadecyltrimethylammonium bromide (Murata et al. 1999). Nucleotide sequencing was conducted using Big-Dye terminator FS core kit and ABI prism 377 auto-sequencer (PerkinElmer Japan, Urayasu). Sequence data were analyzed using the computer software GENETIX-Mac ver 11.0 (Software Development, Tokyo).

## Construction of T-DNA binary vectors

See Table 1 for a description of the plasmids used. A binary vector was constructed based on pIGA0621-EGFP, pLC1-hph, and Mlp5. The *Hin*dIII-*Eco*RI fragment of pIGA0621-EGFP, which contain P*35s*-*Ω*-*egfp*-T*nos* for the expression in *Populus* sp. plants, was cloned into pUC18, generating pHHM181. *dsred2* was newly amplified by PCR with a set of primers, 5′-GGC*TCTAGA*ATGGCCTCCTCCGAGAAC GTCATCACCGAG-3′ and 5′-AAACGC*GAGCTC*CTACA GGAACAGGTGGTGGCGGCCCTC-3′ ($T_a$=70°C), and the plasmid pDsRed2 as a template. The amplified fragment was digested with the restriction endonucleases, *Xba*I and *Sac*I (sequences italicized in the primers), cloned into the appropriate restriction sites of pBluescript II SK+, and then, verified by nucleotide sequencing. *Ω*-*egfp* of pHHM181 was replaced with the *dsred2* fragments by using the restriction sites, generating the plasmid, pHHM186, carrying P*35s*-*dsred2*-T*nos*.

P*gpd*-5′*gpd* containing the sequences from 224 bp of the first exon through 8 bp of the fourth exon from *C. hirsutus* was amplified by PCR with primers 5′-TACCC*AAGCTT*AG AGGCGAGAGCGGACG-3′ and 5′-TCTAG*TCTAGA*GATA CGACCTTGAAAAGGATGAG ($T_a$=56°C), and the plasmid Mlp5 as a template (Orihara et al. 2005). The amplified fragment was digested with the restriction endonucleases *Hin*dIII and *Xba*I (sequences italicized in the primers), cloned into the appropriate restriction sites of pBluescript II SK+, and then, verified by nucleotide sequencing. P*35s* of pHHM186 was replaced with the P*gpd*-5′*gpd* fragment by using the restriction sites, generating pHHM188 carrying P*gpd*-5′*gpd*-*dsred2*-T*nos*. The *Hin*dIII-*Eco*RI fragment containing P*gpd*-5′*gpd*-*dsred2*-T*nos* of pHHM188 was transcloned into pBluescript II SK+, generating the plasmid pHHM190. Then, the *Eco*RV-*Mun*I fragment containing P*ras*-*hph*-T*priA* from pLC1-hph was inserted into the *Sma*I site of pHHM190 after filling the *Mun*I site of the insert with KOD DNA polymerase (Toyobo), generating the binary vector pHHM192. The *Kpn*I fragment containing P*ras*-*hph*-T*priA* and P*gpd*-5′*gpd*-*dsred2*-T*nos* of pHHM192 was then transcloned into pIGA0200, a vector that contains the cloning site within T-DNA, generating pHHM194 (Fig. 1, Table 1). As a control, pHHM203 was constructed by inserting only the blunt-ended *Eco*RV-*Mun*I fragment containing P*ras*-*hph*-T*priA* into the blunt-ended *Hin*dIII-*Xba*I site of pIGA0200 (see above for filling ends).

## Culture of *A. tumefaciens* for agroinfection

*A. tumefaciens* carrying a plasmid was precultured on SGc agar containing Spc at 28°C. Bacterial cells were inoculated into a SGc liquid medium containing Spc and Acs, grown in a 28°C rotary shaker to the mid-exponential growth phase ($OD_{600}$=0.5–0.9), and used for transformation.

## Transformation of *S. grevillei* by agroinfection

A method described by Hanif et al. (2002) was modified. *S. grevillei* mycelia were grown to the diameter of ca 1 cm on a nylon membrane (1.5×1.5 cm, pore size=20 μm) overlaid on the MMN-V8 agar at 25°C. Mycelia on the membrane was dipped into the culture of *A. tumefaciens* carrying a vector for 10 min (see above), and then placed on MMN-V8 agar containing Acs. After incubation at 25°C for 5 days on the agar plate containing Acs, mycelia on the membrane were washed once in sterile 100-μM Cef solution and placed on MMN-V8 containing Hyg and Cef, and incubated at 25°C. As a control, *S. grevillei* mycelia were treated with *A. tumefaciens* AGL1 and EHA105 without a plasmid, in the same way as done for agroinfection.


**Fig. 2** Expression of the Hyg$^r$ trait in *S. grevillei*. *1–2 S. grevillei* Sg-i3 carrying pHHM194 after agroinfection, *3 S. grevillei* Sg-i3 dipped into the culture of *A. tumefaciens* without the plasmid. The photo was taken 20 days after agroinfection

### Detection of DsRed2

Expression of DsRed2 in hyphae was examined under a fluorescent microscope-Leica DMI4000B with the TRITC filter (excitation filters at 580/20 nm, dichroic miller, 595 nm, and emission filters at 630/55 nm). Photos were taken under ×20 objective lens with the exposure time of 450 ms.

### Identification of recombinant DNA in *S. grevillei*

Total DNA from *S. grevillei* strains was analyzed by PCR. *dsred2* was identified by primers used for the construction of the vectors (see above), and *hph*, by a set of primers, 5′-GAGATATGAAAAAGCCTGAACTCACCGC-3′ and 5′-CGGTTTCCACTATCGGCGAGTACTTCT-3′ ($T_a$=60°C) (Ogawa et al. 1998).

## Results

Transformation of *S. grevillei* Sg-i3 was carried out with three replicates, and such a set of experiment was repeated twice independently. In the first round of transformation, giving rise to a total of five transformants out of 12 replicates, one Hyg$^r$ colony was generated with *A. tumefaciens* AGL-1 carrying pHHM194 (AGL-1/pHHM203), two Hyg$^r$ with EHA105/pHHM194, and one Hyg$^r$ of each with AGL-1/pHHM203 and EHA105/pHHM203 (Fig. 2). In the second round of the experiment, producing nine transformants out of 12 replicates, two Hyg$^r$ colonies were obtained with AGL-1/pHHM194, two Hyg$^r$ with EHA105/pHHM194, two Hyg$^r$ with AGL-1/pHHM203, and three Hyg$^r$ with EHA105/pHHM203. In summary, both *A. tumefaciens* AGL1 and EHA105 were capable of transferring the T-DNA vectors to

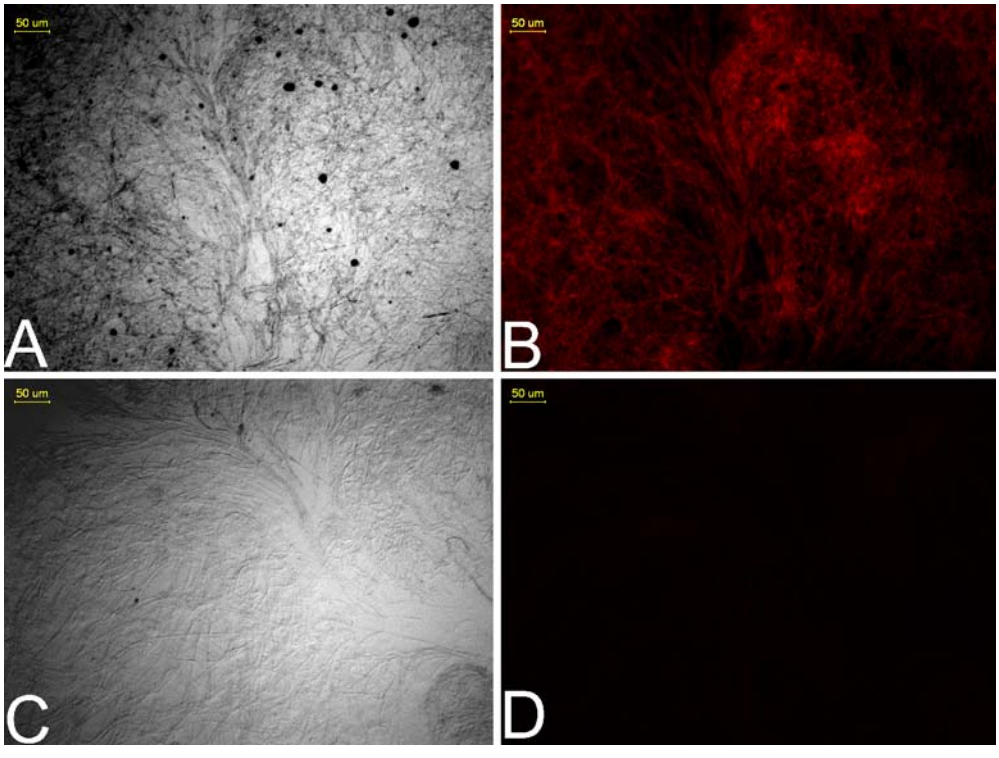


**Fig. 3** Expression of DsRed2 in *S. grevillei*. The analysis was carried out with a fluorescent microscope Leica DMI4000B (×20 objective). **a**, **b** and **c**, **d** *S. grevillei* Sg-i3 carrying pHHM194 and pHHM203, respectively. **a**, **c** Views under phase contrast microscopy. **b**, **d** Views of mycelia shown in **a** and **c**, respectively, with the TRITC filter

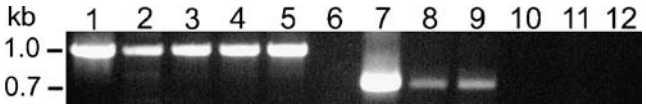


**Fig. 4** PCR analysis of total DNA prepared from the recombinants of *S. grevillei* Sg-i3. Data representing results consistently observed are given. *Lanes 1–6* PCR targeting *hph* (=1071 bp), *lanes 7–12* PCR targeting *dsred2* (=690 bp), *lanes 1 and 7* pHHM194, *lanes 2 and 8*, $Hyg^r/DsRed2^+$ recombinant generated by agroinfection with AGL-1/pHHM194, *lanes 3 and 9* $Hyg^r/DsRed2^+$ recombinant generated by agroinfection with EHA105/pHHM194, *lanes 4 and 10* $Hyg^r/DsRed2^-$ recombinant generated by agroinfection with AGL-1/pHHM203, *lanes 5 and 11* $Hyg^r/DsRed2^-$ recombinant generated by agroinfection with EHA105/pHHM203, *lanes 6 and 12* wild-type. Molecular markers (kb) are given

*S. grevillei* Sg-i3 to the same extent, and $Hyg^r$ mycelia were generated from *S. grevillei* Sg-i3 through agroinfection in ca 58% of the total replicates.

The $Hyg^r$ mycelia were grown to the diameter of ca 2.5 cm and then transferred to the fresh selection agar plate and incubated for further selection. Microscopic analysis revealed that DsRed2 was expressed in the hyphae of all $Hyg^r$ recombinants carrying pHHM194 examined to be clearly distinguishable from the wild types or ones carrying pHHM203 (Fig. 3). PCR analysis confirmed the presence of both *hph* and *dsred2* in the $Hyg^r/DsRed^+$ recombinants and only *hph* in $Hyg^r/DsRed^-$ recombinants, while such DNA segments were not detected in the wild types (Fig. 4). Under the same assay conditions, $Hyg^r/DsRed^+$ and $Hyg^r/DsRed^-$ recombinants were generated with pHHM194 and pHHM203, respectively, from other strains of *S. grevillei* in our laboratory collection.

## Discussion

We demonstrated, for the first time, the expression of autofluorescent proteins in *Suillus* spp. Previously, Hanif et al. (2002) reported the *Agrobacterium*-mediated transformation of *S. bovinus* with the binary vector pBGgHg carrying P*gpd*-*hph*-T*35s* and P*gpd*-*egfp*-T*35s*. Their system, however, conferred only $Hyg^r$ but not $EGFP^+$ on the symbiont. In fact, pBGgHg, which was constructed using P*gpd* from the common mushroom, *Agaricus bisporus*, as a *cis*-regulator, did not confer EGFP expression on the homologous system, either (Chen et al. 2000). In addition to the difference of the fungal species, the types of autofluorescent proteins and origins of *cis*-regulators used in transformation, there are two major differences between their system and ours. Unlike their pBGgHg, our pHHM194 was constructed using two different promoters and terminators to drive *hph* and an autofluorescent protein gene, respectively, so that no homologous regions occurred within the vector. The presence of a homologous region in a given plasmid often causes some DNA rearrangement especially during the maintenance of the plasmid in a $RecA^+$ bacterium, such as wild-type *A. tumefaciens* (Lloyd and Low 1996; Roth et al. 1996). We also designed to use P*gpd*-5′*gpd* region containing the sequences from 224 bp of the first exon through 8 bp of the fourth exon (Orihara et al. 2005) rather than P*gpd* alone. Autofluorescent proteins and other recombinant proteins are generally expressed in wood rotting and saprophytic basidiomycetes, including *A. bisporus*, without a selection pressure when a given gene was fused with *cis*-regulatory sequences carrying introns and constitutive promoters (Burns et al. 2005; Lugones et al. 1999; Ma et al. 2001; Orihara et al. 2005).

One of major issues that needs to be clarified in the research of ectomycorrhizas is how heterologous fungal genets interact with each other during colonization, symbiosis, and fruit body production (Dahlberg 1997; Dahlberg and Stenlid 1994; Murata et al. 2005). Once recombinants of the model symbionts that express various autofluorescent proteins are generated, we may be able to analyze such physical interactions among a given fungal population in vitro. It may be interesting to examine if "coordination" among genets within a population as observed in biofilm in a wide range of microorganisms also occurs in ectomycorrhizal symbionts (Babasaki et al. 2003; Peabody et al. 2000; Ramage et al. 2005; Waters and Bassler 2005). Autofluorescent proteins have been used as powerful tools to analyze in situ in real time host–parasite interactions *in planta* and microbe–microbe interactions in natural environment (Larrainzar et al. 2005).

Unlike the protoplast-mediated transformation, the *Agrobacterium*-transformation that introduces a vector into mycelia cannot confer clonal insertion mutants originated from a single cell, unless fungal mutant lines are regenerated from spores as in the case of plant mutant lines regenerated from recombinant seeds (Clough and Bent 1998; Combier et al. 2004; Feldmann and Marks 1987). However, the *Agrobacterium*-mediated transformation system with a binary vector allows functional analysis of a given gene through the expression of both a selection marker and a gene of interest in dikaryons, hyphae that are composed of cells with two haploid nuclei and generally observed in homobasidiomycetes (Alexopoulos et al. 1996). Recently, Combier et al. (2004) isolated and characterized using protoplast-mediated transformation nonmycorrhizal insertion mutants of monokaryons of *Hebeloma cylindrosporum*, a symbiont that colonizes shortly, spreads through the dispersal of spores very frequently, and is able to form mycorrhizas as haploid monokaryons. We are currently attempting to develop an *Agrobacterium*-mediated transformation system to generate clonal insertion mutants from spores of *Lyophyllum shimeji*, an ectomycorrhizal homobasidiomycete that can be

cultivated in a spawn to produce commercially valuable fruit bodies (Kawai 1997; Ohta 1994). Technical development based on the present achievement may facilitate functional analysis of ectomycorrhizal symbiosis in terms of genetics and molecular biology, research areas that have been hampered for years due to the lack of technical tools.

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