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SHORT COMMUNICATION

Agrobacterium tumefaciens-mediated genetic transformation of the ectomycorrhizal fungus *Laccaria laccata*

Mariane Paludetti Zubieta · Irene da Silva Coelho · Marisa Vieira de Queiroz · Elza Fernandes de Araújo

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Abstract Ectomycorrhizal fungi form symbiotic relationships with the roots of most tree species and support increased forest health and productivity. The ectomycorrhizal fungus *Laccaria laccata* was successfully transformed through *Agrobacterium tumefaciens*-mediated transformation (ATMT). We observed a transformation efficiency of 80 %, with 100 % of the assessed transformants exhibiting mitotic stability after five passages in culture medium without the selective agent. The amplification of the 626-bp DNA fragment corresponding to the hygromycin B phosphotransferase (*hph*) gene and a Southern blotting analysis showed that the assessed transformants had integrated the T-DNA into the genome, confirming the success of the ATMT procedure. Single T-DNA integrations were detected in five of the eight transformants assessed by Southern blotting, and the hybridization pattern confirmed that the integration occurred randomly in the genome of the fungus. The development of an efficient ATMT system that allows the integration of single T-DNA copies into the *L. laccata* genome makes functional genetic studies in this ectomycorrhizal fungus feasible.

Keywords *Agrobacterium tumefaciens* · Genetic transformation · Hygromycin B resistance · Insertional mutagenesis · *Laccaria laccata* · T-DNA

M. P. Zubieta · M. V. de Queiroz · E. F. de Araújo (✉)
Microbiology Department, Federal University of Viçosa, CEP 36570-000 Viçosa, Brazil
e-mail: ezfa@ufv.br

I. da Silva Coelho
Veterinary Microbiology and Immunology Department, Rural Federal University of Rio de Janeiro, CEP 23890-000 Seropédica, Brazil

Laccaria laccata has been reported as a common mychorrizal fungus of angiosperms and gymnosperms (Giachini et al. 2004). Fungal species belonging to this genus exhibit the ability to form ectomycorrhizae with greenhouse plants (Southworth et al. 2009). This feature, along with vegetative growth from basidiospore germination and the easy handling of the culture, allows for the use of *L. laccata* in ectomycorrhizal interaction studies (Osmundson et al. 2005).

The establishment of ectomycorrhizal symbiosis is activated by signals produced by fungal hyphae and plant roots. Physiological and morphological changes observed during ectomycorrhizal development are concurrent with changes in gene expression in both symbionts, starting before any physical contact (Coelho et al. 2010). Random insertional mutagenesis of both partners using transforming DNA (T-DNA) is a strategy that might allow the targeting of genes responsible for the formation of ectomycorrhizae. In turn, in those fungal strains that have lost the ability to form ectomycorrhizae, the disrupted genes can be identified because they are tagged by the T-DNA (Sugui et al. 2005).

An efficient transformation protocol should be developed for insertional mutagenesis studies. *Agrobacterium tumefaciens*-mediated transformation (ATMT) has been used to transform a wide variety of filamentous fungi (Nyilasi et al. 2005; Hanying et al. 2012; Nizam et al. 2012) and has several advantages over other transformation techniques in that this method can generate transformants with single genomic integrations and does not require protoplast isolation for successful completion (Michielse et al. 2005).

In the present study, we describe an efficient protocol for the ATMT of *L. laccata* vegetative mycelium. *L. laccata* isolate belongs to the Fungal Collection of Mycorrhizal Associations Laboratory of the Microbiology Department of the Federal University of Viçosa, and the hypervirulent *A. tumefaciens* strain AGL-1 that harbors the binary vector pBGgHg was generously provided by Dr. C. Peter Romaine

(Department of Plant Pathology, Pennsylvania State University, University Park, PA 16802, USA). The pBGgHg vector contains the *Escherichia coli* hygromycin B phosphotransferase (*hph*) gene and the *Aequorea victoria*-enhanced green fluorescent protein (*egfp*) gene in between the right and left border sequences of *Agrobacterium* T-DNA. Each gene is under *Agaricus bisporus gpd* promoter (Chen et al. 2000).

To evaluate the minimal concentration of hygromicin B that inhibited the growth of an *L. laccata* wild type strain, the fungal culture was grown on MNM medium (Marx 1969) supplemented with different concentrations of hygromycin B (μg/ml): 40, 80, 120, 160, 200 and one negative control that did not contain antibiotic. Three repetitions were performed for each concentration, and the plates were incubated at 28 °C for seven days. The mycelium growth *of L. laccata* was completely inhibited by concentrations of hygromycin B above 120 μg/ml (Fig. 1A). Thus, the selection of transformants in the ATMT experiments was performed with 120 μg/ml hygromycin B.

The ATMT procedure was based on a previously described protocol (Kemppainen et al. 2005; Kemppainen and Pardo 2011) with several modifications. *L. laccata* inocula were let to grow on MNM medium at 28 °C for seven days; then agar plugs (5 mm in diameter) were removed from the edges of fungal colonies and transferred to plates containing cellophane membranes on MNM medium. After three days at 28 °C, fungal colonies grown from the agar plugs were transferred to new plates containing cellophane membranes on induction medium with or without 200 μM of acetosyringone (AS) (Sigma, United States). *A. tumefaciens* culture was prepared as follows. Briefly, an *A. tumefaciens* AGL-1 strain harboring pBGgHg (Chen et al. 2000) was grown in 20 ml of LB liquid medium supplemented with 50 μg/ml kanamycin at 28 °C with shaking (150 rpm) for 48 h. The culture was diluted to an optimal OD_{660} of 0.15 with induction medium (Bundock et al. 1995) containing 200 μM acetosyringone. Next, *A. tumefaciens* was pre-cultured at 28 °C for 4 h with gentle shaking at 150 rpm to an OD_{660} of 0.4. The fungal colonies were then inoculated with 10 μl of *A. tumefaciens* culture, and the co-cultivation plates were incubated at 22 °C for five days in the dark; cellophane membranes containing the fungal colonies were transferred to new plates containing the selection medium (MNM medium supplemented with 120 μg/ml hygromycin B and 200 μM of cefotaxime) to select for *L. laccata* transformants while inhibiting the growth of *A. tumefaciens* and kept at 28 °C for 10 days. All experiments were performed four times with 100 agar plugs each one.

Fungal-resistant sectors formed at the edges of *L. laccata* colonies were individually harvested and isolated for vegetative propagation in new plates containing selective medium. Fungal colonies that maintained their growth were considered as stably transformed strains.

To evaluate mitotic stability of the transformants, 70 randomly selected transformants were grown in MNM medium

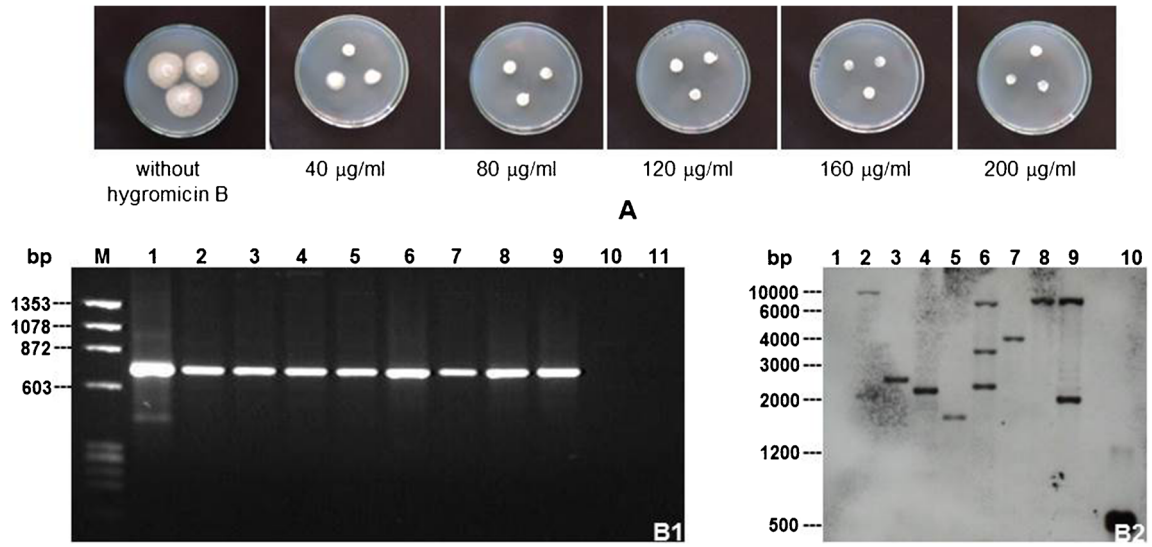

Fig. 1 (**A**) Resistance test of wild type *Laccaria laccata* to increasing concentrations of hygromycin B. (**B**) Molecular analysis of obtained transformants. **B1**: PCR confirmation of the presence of the *hph* gene in hygromycin-resistant *Laccaria laccata* transformants. The PCR analysis was carried out using genomic DNA and the hph1 and hph2 primers, which amplified a 0.696-kb fragment. M: DNA molecular size marker, ϕX174/*Hae*III, positive control with the pBGgHg vector (lane 1), randomly selected hygromycin-resistant *Laccaria laccata* transformants (lane 2–9), wild type DNA (lane 10) and negative control (lane 11). **B2**: Autoradiography analysis by Southern blotting. M: DNA molecular size marker, GeneRuler™ DNA Ladder Mix (Thermo Scientific, United States), wild type DNA (lane 1), DNA from putative transformants TN1, TN75, TN 151, TN196, TN283, TN329, TN331, and TN35 (lanes 2–9, respectively), pBGgHg DNA from *A. tumefaciens* (lane 10)

without hygromycin B for five generations (collected every seven days) and transferred back into MNM medium with hygromycin B.

DNA was isolated from wild type (control) strain and eight mitotically stable transformants as described by Schäfer and Wöstemeyer (1992) and Junghans et al. (1998). The extraction of plasmid DNA from *A. tumefaciens* was performed using the GeneJET Plasmid Miniprep Kit (Fermentas, United States) according to the manufacturer's instructions.

The DNA samples obtained were used in PCR reaction and Southern blotting. For the PCR reaction, the primers hph1 (5′-TTCGATGTAGGAGGGC GTGGAT-3′) and hph 2 (5′-CGCGTCTGCTGCTCCA TACAAG-3′) were used to detect the presence of the phosphotransferase gene (*hph*). For Southern analysis, 3 μg of genomic DNA was cleaved with *Eco*RI restriction enzyme. Since this enzyme does not have a cleavage site in the *hph* gene sequence, the number of the bands that hybridize with the *hph* probe (626-pb) is a marker of the T-DNA copies integrated into the host genome. Southern blotting was performed according to Southern (1975); the probe was labelled using the PCR DIG Probe Synthesis Kit (Roche, Germany), and the hydridizing bands were detected using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany) according to the manufacturer's instructions.

To detect fluorescence emission in the mycelium of the transformants, fragments of mycelia were collected, arranged on a glass slide, and analysed under fluorescent light. The images were visualized using the Olympus BX-60 microscope with an excitation filter from 460 to 480 nm and edited with the Image-Pro Plus version 4.0 program.

Three hundred twenty-two of the 400 mycelium-containing culture medium disks that were placed in contact with the *Agrobacterium* for the four experiments (100 disks per experiment) exhibited mycelial growth in the selection medium, representing an 80.5 % T-DNA transfer efficiency (Table 1). The transformation efficiency obtained in ATMT experiments of *L. laccata* was higher as compared with the transformation of *L. laccata* using PEG-$CaCl_2$ and is one of the highest ever described for ectomycorrhizal fungi transformed by *A. tumefaciens* (Barrett et al. 1990; Pardo et al. 2005; Kemppainen et al. 2005; Murata et al. 2006). Transformants were not observed in co-culture plates without AS, as observed in ATMT of fungi-like *Aspergillus awamori*, *Neurospora crassa*, and *Agaricus bisporus* (De Groot et al. 1998).

Since repetitive passages on media without the selective agent did not alter the capacity of the mycelial strains to grow when they were re-transferred onto MNM medium supplemented with hygromycin, the mitotic stability of the transformants was confirmed. Mitotically stable transformants were also found in other experiments using ATMT (Kemppainen and Pardo 2011; Zhang et al. 2013). The transformants did not display any phenotypic alteration when grown on MNM, regardless of the presence of hygromycin B in the medium.

Table 1 Transformation efficiencies (%) in four independent experiments of ATMT of *Laccaria laccata*

Experiment	Number of agar plugs containing mycelia	Number of transformants	Transformation efficiency (%)[a]
1	100	99	99
2	100	89	89
3	100	66	66
4	100	68	68
Total	400	322	80.5±12.5[b]

[a] Transformation efficiency (%) = Number of transformants / Number of agar discs containing mycelia

[b] The result is the mean from four independent experiments ± standard error of the mean.

Differently from the wild type strain, the eight putative transformants displayed a 626 bp-long fragment when their DNA was PCR amplified with the primer pair specific to the *hph* (Fig. 1B1).

To construct a collection of T-DNA tagged *L. laccata* strains it is recommendable that T-DNA integrates only once and randomly into the genome. The hybridization patterns indicated that the T-DNA integration occurred in different regions of the genome either once or even up to 3 times. This result illustrates the advantage of choosing this (Fig. 1B2) technique over other transformation methods.

The transformants were evaluated for fluorescence emission due to the presence of the *egfp* gene in the vector pBGgHg between the T-DNA borders. However, this ability was not observed. Strains of *Agaricus bisporus* transformed with the plasmid pBgGHg did not show fluorescence either. According to the literature, heterologous expression of *egfp* has not been observed successfully in some organisms, due to incorrect mRNA processing and differences in codon preferences between the organisms involved (Haseloff et al. 1997; Pöggeler et al. 2003).

The results of the present study provide the first evidence for genetic transformantion of *L. laccata* by *A. tumefaciens*. This step is crucial for the identification and manipulation of *L. laccata* genes related to symbiosis.

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