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ORIGINAL PAPER

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Agrobacterium-mediated transformation of the ectomycorrhizal symbiont *Laccaria bicolor* S238N

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Abstract The development of an efficient transformation system is required to alter the expression of symbiosis-regulated genes and to develop insertional mutagenesis in the ectomycorrhizal basidiomycete *Laccaria bicolor* S238N. Vegetative mycelium of this fungus was transformed by *Agrobacterium tumefaciens*-mediated gene transfer. The selection marker was the hygromycin resistance gene of *Escherichia coli* (*hph*) under the control of the *gpd* promoter from *Agaricus bisporus* and the CaMV 35S terminator as part of the T-DNA. PCR amplification of *hph* and Southern blot analyses showed that the genome of the hygromycin-resistant transformants contained the cassette. The later proved mostly single copy and random integration of part of the transgene into the fungal genome. *A. tumefaciens*-mediated gene transfer should facilitate future development of insertional mutagenesis, targeted gene disruption and RNA interference technology in *L. bicolor*.

Keywords *Laccaria* · *Agrobacterium* · Transformation · Ectomycorrhiza

Introduction

Characterization of the primary genetic traits controlling symbiosis development and its metabolic activity, such as nutrient scavenging, transport and assimilation will help in understanding the ecological role of ectomycorrhizal symbiosis (Martin 2001; Wiemken and Boller 2002). To understand the complex interactions that control ectomycorrhizal development, large scale EST sequencing, cDNA array analysis of gene expression and proteomics have been developed on several symbiotic associations (Laurent et al. 1999; Voiblet et al. 2001; Podila et al. 2002; Peter et al. 2003; Johansson et al. 2004; Duplessis et al. 2005). Comparisons of gene expression in free-living partners and symbiotic tissues revealed significant differences in the expression levels and have identified about 150 symbiosis-regulated fungal genes (Martin et al. 2004).

Laccaria bicolor is a common ectomycorrhizal symbiont of many temperate forest trees, among them *Populus trichocarpa*, the first perennial plant to have its genome sequenced. In nature and in the laboratory *L. bicolor* usually grows as a heterokaryon composed of two different nuclei, each of a different mating type, but axenic haploid strains isolated from spores are available (Di Battista et al. 1996). Currently, there is sequence from approximately 2,000 ESTs and random genomic fragments deposited in GenBank (Podila et al. 2002; Peter et al. 2003). The Joint Genome Institute (JGI) is currently sequencing the genome of the ectomycorrhizal basidiomycete *Laccaria bicolor* S238N (Lammers et al. 2004; Martin et al. 2004).

So far the generation of null mutants for these genes has been hampered by the lack of efficient transformation systems. Screening of *Laccaria* transformants obtained using protoplast-PEG-based transformation (Barrett et al. 1990) and particle-bombardment-mediated method (Bills et al. 1999) showed multiple copy integrations, which is a constraint for subsequent molecular characterization of tagged genes.

A. tumefaciens-mediated transformation, besides being used for a long time to transform natural plant host (Tinland 1996), has also succeeded in transformation of human cells (Kunik et al. 2001), yeast (Bundock et al. 1995), filamentous fungi (de Groot et al. 1998) and other ectomycorrhizal basidiomycetes (Pardo et al. 2002; Hanif et al. 2002; Combier et al. 2003). Moreover, transformation with this

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method is mainly the result of single-copy integration events (Hellens et al. 2000).

Here we report the successful *A. tumefaciens*-mediated transformation of the vegetative mycelium of *L. bicolor* to hygromycin resistance. Southern blot analysis indicates that most transformants contain a single copy of the transgene integrated at random.

Materials and methods

Strains and plasmids

L. bicolor (Maire) Orton isolate S238N was described previously (Di Battista et al. 1996). The dikaryotic vegetative mycelium was maintained at 22°C in the dark on agar P5 medium (Martin et al. 1998). *Agrobacterium tumefaciens* strains LBA1100 and AGL1 were kindly supplied by Paul Hooykaas (Leiden University, Netherlands) and by Peter Romaine and Carl Schagnhaufer (Pennsylvania State University, USA), respectively. Voucher specimens are kept in the Universidad Nacional de Quilmes Culture Collection (Argentina). Plasmid pBGgHg (Chen et al. 2000) provided by Peter Romaine and Carl Schagnhaufer was used in this study because it has been proved useful in transformation of other homobasidiomycetous ectomycorrhizal fungi (Hanif et al. 2002; Combier et al. 2003). This plasmid consists of a pCAMBIA 1300 backbone with an *Escherichia coli* hygromycin resistance cassette (*hph*) driven by the *Agaricus bisporus gpd* promoter and terminated by the CaMV 35S terminator (Chen et al. 2000). The vector was introduced into *A. tumefaciens* LBA1100 and AGL-1 strains by electroporation (Pardo et al. 2002).

T-DNA transfer

A. tumefaciens-mediated transformation of *L. bicolor* S238N was done as described (Pardo et al. 2002). Briefly, fungal colonies were grown from several 2-mm agar plugs on cellophane membranes on P5 media at 22°C in the dark during 5 days and then transferred to P20-induction plates [P5 media with lower sugar content (glucose 2 g l^{-1} as the sole carbon source) supplemented by 40 mM MES, 0.5% glycerol and 200 μM acetosyringone (AS) (Aldrich, Milwaukee, WI, USA) at pH 5.3]. The colonies were then inoculated with 50 μl of an induced *A. tumefaciens* (pBGgHg) culture prepared according to Pardo et al. (2002). The co-cultivation plates were incubated at 22°C for 5 days in the dark; cellophane membranes containing the fungal colonies were transferred to new plates containing the selection medium [per litre: P5 media supplemented by 100 μg ml^{-1} cefotaxime, 100 μg ml^{-1} ampicillin and 100 μg ml^{-1} tetracycline for killing *A. tumefaciens* cells, and 300 μg ml^{-1} of HygroGold hygromycin (Invitrogen, Carlsbad, CA, USA) for selection of *L. bicolor* transformants] and kept at 22°C for 7–10 days in the dark.

Resistant colonies were individually harvested and isolated for vegetative propagation in P5 medium containing 300 μg ml^{-1} hygromycin. Four experiments, two using *A. tumefaciens* LBA100 and two using *A. tumefaciens* AGL-1 as donor strains, respectively, were done in six replicates each with 15 fungal colonies per Petri dish (9 cm diameter). Controls using non-transformed wild-type mycelium were always included.

Mycelium from the hygromycin-resistant (after three rounds of selection) and wild-type strains grown for 20 days on cellophane membranes on P5 media, with or without 150 μg ml^{-1} of hygromycin, respectively, was frozen in liquid nitrogen, ground to a fine powder, and genomic DNA was extracted with the DNeasy Plant Mini Kit (Qiagen, Germany). The *hph* resistance gene was amplified by PCR with primers HPH-F (5′-AAGCCTGAACT CACCGCGAC-3′) and HPH-R (5′-CTATTCCTTTGCCC TCGGAC-3′), which give rise to an approximately 1-kb fragment. About 50 ng of genomic DNA and about 1 ng of pBGgHg plasmid DNA, respectively, were used as template. The PCR program was as follows: initial denaturation at 94°C for 2 min, 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, and a final extension at 72°C for 5 min. The amplification products were purified with the Wizard PCR preps DNA purification system (Promega, Madison, WI, USA) and sequenced with the primer HPH-F, the CEQ Dye Terminator Cycle sequencing kit and the CEQ2000XL automated sequencer (Beckman Coulter, Fullerton, CA, USA). To prove the absence of contaminating *A. tumefaciens* cells within the resistant fungal colonies a control PCR was done with primers VIRA-F (5′-ATGAATGGAAGGTATTCACCG-3′) and VIRA-R (5′-GTTTTTGGAGCATGTCGAGTT-3′) specific for the *virA* gene located in the plasmid Ti. PCR conditions were as used for *hph* except that the annealing temperature was 55°C and 1 μl of an overnight *Agrobacterium* culture was used as positive control. This PCR should be negative in truly transformed fungi but positive if *A. tumefaciens* cells were contaminating the mycelium. Southern blot analysis was done by using an approximately 1-kb *hph* fragment as a probe, amplified from pBGgHg by PCR with HPH-F and HPH-R primers under the above PCR conditions. The PCR fragment was cleaned with the Wizard PCR preps DNA purification system (Promega) and labelled with alkaline phosphatase for chemiluminescent detection with the AlkPhos Direct Labelling and Detection System with CDP-Star (Amersham Biosciences, UK) according to the manufacturer's protocol. Genomic DNA (10 μg) from control and putative transformed mycelium was digested with *Eco*RV (Promega), which cuts once within the T-DNA but outside of the *hph* selection marker, and blotted onto Hybond N^+ membrane (Amersham Biosciences) by alkaline transfer and baking according to the manufacturer's protocol.

Results and discussion

To test the *A. tumefaciens*-mediated transformation of *L. bicolor*, vegetative mycelium was co-cultivated with *A. tumefaciens* LBA1100 or AGL-1 containing pBGgHg.

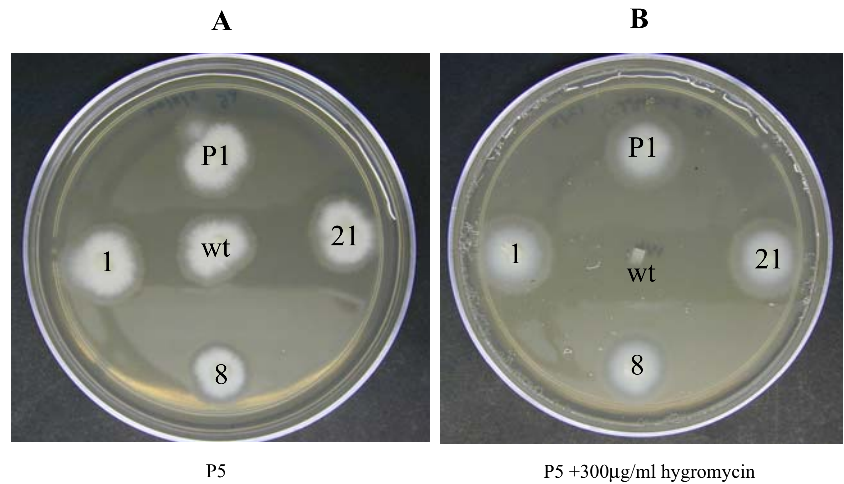

Fig. 1 *L. bicolor* transformants (clones *P1*, *1*, *8* and *21*) and wild type (*wt*) grown on P5 media (**a**) or P5 plus 300 µg of hygromycin/ml (**b**) at 22°C during 7 days. Clones 1, 8 and 21 derived from co-cultivation of *L. bicolor* with *A. tumefaciens* AGL-1 and clone P1 from co-cultivation with *A. tumefaciens* LBA1100

This construct was efficiently used for transformation of other ectomycorrhizal basidiomycetes (Hanif et al. 2002; Combier et al. 2003). In addition, the resistance level of *L. bicolor* to hygromycin was in a range allowing efficient selection of transformants. Co-cultivation of *L. bicolor* with *A. tumefaciens* (pBGgHg) resulted in the formation of small hygromycin-resistant colonies after 7–10 days on selective media (Fig. 1).

After three rounds of selection, PCR amplification using the HPH-F and HPH-R primers indicated that the hygromycin-resistant colonies contained the *hph* sequence (Fig. 2) (confirmed by sequencing) and thus were actually transformed. The efficiency of transformation averaged 10 and 55% when LBA1100 and AGL-1 strains were used as donors, respectively. Differences among *A. tumefaciens* strain efficiencies are well known and the appropriate combination of selection marker, binary vector and *Agrobacterium* strain is critical (Hellens et al. 2000). Transformed fungal DNA was proved to be free of *A. tumefaciens* DNA, as these samples were always negative in a PCR targeted to the *virA* gene, which is located in the plasmid Ti.

To determine the number of different integrations of the T-DNA, genomic DNA from transformants was digested with *Eco*RV and then probed with the 1-kb *hph* fragment from pBGgHg (Fig. 3). *Eco*RV cuts once within the T-DNA and the probe hybridizes to only one of the resulting fragments, thus multiple hybridizing bands would be indicative of multiple T-DNA integration events. By this criterion, transformants mostly contained single copies of T-DNA and this integration event occurred at random. The results presented in Figs. 1, 2 and 3 are representative of about 100 individual transformants from four independent experiments, taking into account the percentages of transformation obtained with each *Agrobacterium* strain.

The mitotic stability of part of the transferred T-DNA was tested by growing the transformants in P5 media without selection. In all cases, resistance to hygromycin was retained after this treatment.

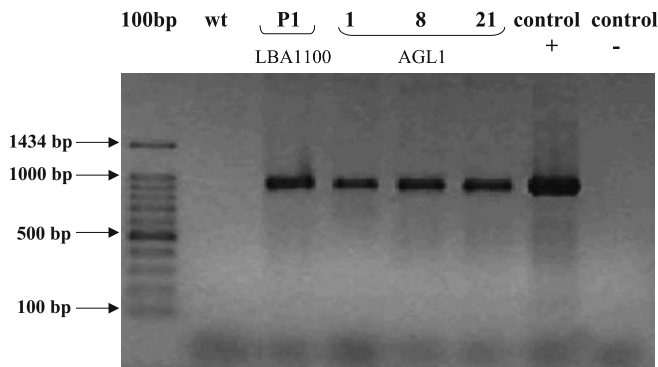

Fig. 2 PCR analysis of *L. bicolor* transformants and wild type targeted to approximately 1-kb *hph* transgene fragment. From *left to right*: molecular size marker 100-bp ladder, *L. bicolor* wild type (*wt*) and transformants (*P1*, *1*, *8* and *21*), positive control with plasmid pBGgHg as template, and negative control without DNA

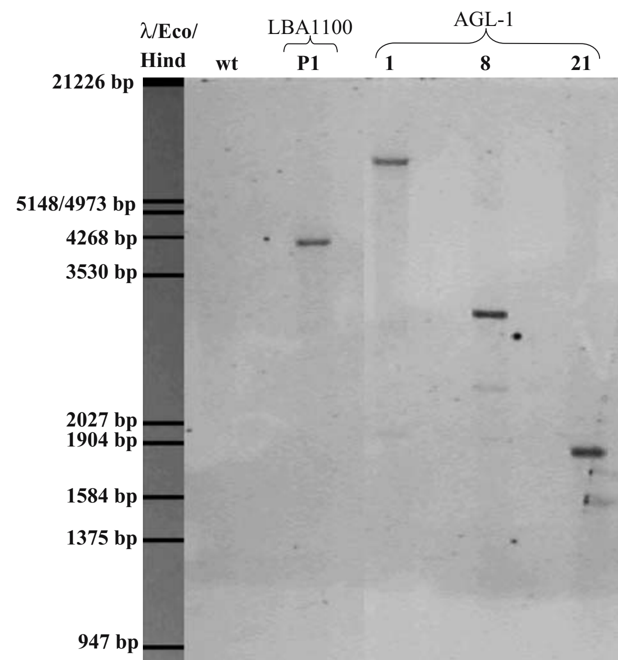

Fig. 3 Southern blot analysis of *L. bicolor* transformants and wild type. Total DNA (10 µg) was digested with *Eco*RV, blotted and probed with the approximately 1-kb *hph* transgene fragment. From *left to right*: molecular size marker λ/*EcoRI*/*HindIII*, *L. bicolor* wild type (*wt*) and transformants (clones *P1*, *1*, *8* and *21*)

A. tumefaciens-mediated transformation is less labor-intensive than protoplast-requiring or biolistic transformation protocols developed for *Laccaria* (Barrett et al. 1990; Bills et al. 1999). Moreover, single and random integration are two critical features requested for the development of large-scale insertional mutagenesis and further recovery of the T-DNA flanking sequences by PCR-based techniques (Combier et al. 2003) or plasmid rescue techniques (Leclerque et al. 2004). The transformation procedure described here is currently used to generate mycorrhiza-defective mutants of *L. bicolor* S238N as recently done for *Hebeloma cylindrosporum* (Combier et al. 2004). A precise understanding of how the symbiosis-regulated genes and proteins function and interact with each other in a cellular context requires the ability to introduce precise alterations within specific components of these genetic networks. Targeted transgenesis and RNA interference, already possible in fungal phytopathogens (Kadotani et al. 2003; Fitzgerald et al. 2004), are not yet available for the study of ectomycorrhizal symbiosis. In this respect, *Agrobacterium*-mediated genetic transformation of the model poplar mycorrhizal symbiont *L. bicolor* will probably be a useful tool to reach these goals in the future.

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