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T-DNA transfer from *Agrobacterium tumefaciens* to the ectomycorrhizal fungus *Pisolithus microcarpus*

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SUMMARY

The model ectomycorrhizal fungus *Pisolithus microcarpus* isolate 441 was transformed by using *Agrobacterium tumefaciens* LBA1100 and AGL-1. The selection marker was the *Shble* gene of *Streptoallotecius hidustanus*, conferring resistance to phleomycin, under the control of the *gpd* gene promoter and terminator of *Schizophyllum commune*. Transformation resulted in phleomycin resistant clones which were confirmed by PCR to contain the resistance cassette. *A. tumefaciens*-mediated gene transfer would allow the development of RNA interference technology in *P. microcarpus*.

Key words: *Pisolithus*, fungi, *Agrobacterium*, ectomycorrhiza

RESUMEN

Transferencia de T-DNA de *Agrobacterium tumefaciens* al hongo ectomicorrícico *Pisolithus microcarpus*. El hongo ectomicorrícico modelo *Pisolithus microcarpus* aislamiento 441 fue transformado utilizando *Agrobacterium tumefaciens* LBA 1100 y AGL-1. El marcador de selección fue el gen S*hble* de *Streptoallotecius hidustanus*, el cual confiere resistencia a fleomicina, bajo el control del promotor y terminador del gen *gpd* de *Schizophyllum commune*. La transformación resultó en clones resistentes a fleomicina comprobándose por PCR la presencia del transgen. La transferencia génica mediada por *Agrobacterium* podría permitir el desarrollo de la tecnología de interferencia por ARN en *P. microcarpus*.

Palabras clave: *Pisolithus*, hongos, *Agrobacterium*, ectomicorriza

INTRODUCTION

The fungal genus *Pisolithus* is cosmopolitan in warm temperate regions of the world and forms ectomycorrhizal associations with a wide range of woody plants (6, 18) including members of the *Pinaceae* and *Myrtaceae*. *Pisolithus* spp. has extensively been used in both basic and applied research. The physiological ecology of *Pisolithus* spp. is well studied among ectomycorrhizal taxa, because they grow rapidly in culture and their mycorrhiza are easily established with tree roots under laboratory conditions (5). *In vitro* systems have been used to study symbiosis development, carbon metabolism, nitrogen and phosphorous acquisition and transport, and the ability of this fungus to scavenge nutrients from soil (for a review see 15). Moreover, this species is used in large-scale commercial inoculation programs in forest nurseries worldwide to enhance growth of tree seedlings (10). It is apparent from molecular studies of *P. microcarpus* ectomycorrhiza, that there is a vast complexity of genetic programmes with overlapping expression patterns. This includes the morphogenetic switches of the fungal hyphae, the establishment of novel cell walls and extracellular matrices and the onset of a novel metabolism. Several studies using either EST profiling (21) or a gene array approach (24) have examined the expression of genes in *Pisolithus-Eucalyptus* ectomycorrhizas. Up to 65 symbiosis-regulated *P. microcarpus* genes and proteins have been identified (12, 21, 24). Yet a precise understanding of how these 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. In this respect, targeted transgenesis in ectomycorrhizal fungi is not possible at present. Testing the roles of candidate *Pisolithus* genes in ectomycorrhiza formation requires a routine transformation procedure.

Despite several trials, different direct gene transfer technologies (protoplasting, electroporation, biolistic) were unsuccessful in *Pisolithus* (our unpublished data) although these techniques did allow the transformation of other ectomycorrhizal basidiomycetes (1, 3, 14).

Several fungal species refractory to classical transformation techniques have been transformed by *A. tumefaciens* (7). This system originally developed for plants has been adapted to yeast (4) and filamentous fungi as well (9). Moreover, several ectomycorrhizal basidiomycete species have been amenable to being transformed by *Agrobacterium* by using either a phleomycin resistance gene (20) or hygromycin resistance and green fluorescent protein genes (8, 11). This opens new posibilities for the transformation of *Pisolithus* spp.

In this study we report the obtention of phleomycin resistant mycelial colonies of *P. microcarpus* through the T-DNA transfer from two different strains of *A. tumefaciens* for the first time.

MATERIALS AND METHODS

Fungal and bacterial strains, and in vitro ectomycorrhiza synthesis

Pisolithus microcarpus Coker & Couch isolate 441 (formerly identified as *Pisolithus tinctorius* 441) (17) was used as a recipient strain in cocultivation experiments with *Agrobacterium tumefaciens.* The mycelium was maintained at 22 °C in darkness on agar P5 medium (16). *E. coli* XL-1Blue (Stratagene, CA) was the recipient strain for cloning experiments. *A. tumefaciens* LBA1100 was a gift from Paul Hooykaas (Leiden University) and AGL-1 from Peter Romaine and Carl Schagnhaufer (Pennsylvania State University): these two strains were used for *P. microcarpus* genetic transformation. Voucher specimens are kept in the Universidad Nacional de Quilmes Culture Collection (Argentina). For *in vitro* ectomycorrhiza synthesis, sterilized *Eucalyptus globulus bicostata* seeds were germinated on a low-sugar content agar medium (19) and seedlings were used for inoculation with *P. microcarpus* mycelium (5). After 15 to 20 days of inoculation, mature ectomycorrhizas were obtained.

Plasmid

Plasmid pBIN19-17 was used (2, 20). Briefly, this binary vector contains a chimeric phleomycin resistant gene where the *Shble* (from *Streptoalloteicus hidustanus*) coding sequence was cloned between the promotor and terminator of the gly-ceraldehyde-3-phosphate dehydrogenase gene (*gpd*) from *Schizophyllum commune* (22). The phleomycin resistance box in plasmid pGPhT was a gift from Frank Schuren (TNO Nutrition and Food Research Institute, The Netherlands).The vector was electroporated into *A. tumefaciens* LBA1100 and AGL-1 strains according to (20).

T-DNA transfer

Fungal colonies were grown from several 5 mm agar plugs on cellophane membranes on P5 media (19) at 28 °C and then transferred to P5-induction plates [P5 media with low-sugar content (glucose 2 g l^{-1} as the sole C source) supplemented with 40 mM MES; 0.5% glycerol and 200 µM acetosyringone (AS) (Aldrich, WI) pH 5.3]. After 7 days, the colonies were inoculated with 50 µl of an *A. tumefaciens* [pBIN19-17] culture (20). The co-cultivation plates were incubated at 28 °C for 5 days; cello-phane membranes containing the fungal colonies were transferred to new plates containing the selection medium [P5 medium supplemented with 100 µg ml^{-1} cefotaxime; 100 µg ml^{-1} ampicilline and 100 µg ml^{-1} tetracycline for killing *A. tumefaciens* cells, and 250 µg ml^{-1} of phleomycin (Cayla, Toulouse) for selection of *P. microcarpus* transformants, kept at 4 °C overnight and then shifted to the growing temperature (28 °C) for 2 to 3 weeks.

Resistant colonies were individually harvested and isolated for vegetative propagation in P5 medium supplemented with 250 µg ml^{-1} of phleomycin. Experiments were performed three times by ten replicas for each *A. tumefaciens* strain, using fifteen fungal colonies per 9 cm diam. Petri dish. Negative controls using non-transformed *A. tumefaciens*, non-induced *A. tumefaciens* and wild type *Pisolithus* were always included.

DNA extraction and PCR screening

Following five rounds of selection the mycelium of the phleomycin-resistant and wild type strains was grown for one month on cellophane membranes on P5 medium, with or without phleomycin respectively, frozen in liquid nitrogen and ground to a fine powder. Total DNA was extracted using the DNA Plant Mini Kit (Qiagen, Germany). The presence of the *Shble* chimeric gene conferring resistance to phleomycin was confirmed by PCR using the primers GPDsen and BLEant which amplified a specific 0.5 kb fragment (20). About 100 ng of genomic DNA and ~10 pg of pBIN19-17 plasmid DNA respectively were used as template. The construct identity was confirmed by se-quencing with the Big Dye Terminator Cycle sequencing kit (PE Biosystems, CA) using the primer GPDsen, after purification from the PCR mix with the Wizard^{R}PCR preps DNA purification system (Promega, WI). In order to prove the absence of contaminating *A. tumefaciens* cells within the resistant fungal colonies, a control PCR was carried out with specific primers for pBIN19-17 backbone, annealing outside of the T-DNA, on the kanamycin resistance gene (11). This PCR should be negative in truly transformed fungi but positive if *A. tumefaciens* cells were contaminating the mycelium. In order to know whether DNA in non-transformed *P. microcarpus* was amenable to being amplified, a control PCR targeted to the fungal ribosomal internal transcriber spacer (ITS) was carried out according to a previous publication (20).

Table1. Percentage of transformation of *P. microcarpus* to phleomycin resistance after 5 rounds of selection in 3 independent experiments. Fungal colonies (150 per treatment divided in 10 Petri dishes) where co-cultivated either with *A. tumefaciens* LBA100 or AGL-1 carrying the binary vector pBIN19-17 and induced with AS. Controls using *P. microcarpus* wt, non-transformed *A. tumefaciens* and non-induced *A. tumefaciens* (150 fungal colonies per treatment divided in 10 Petri dishes) were included in each experiment. For details see materials and methods

Experiment	Wt	LBA1100 + AS	LBA1100 (pBIN19-17) + AS	LBA1100 (pBIN19-17) - AS	AGL-1 + AS	AGL-1 (pBIN19-17) + AS	AGL-1 (pBIN19-17) - AS
1	0	0	30	0	0	35	0
2	0	0	33	0	0	40	0
3	0	0	41	0	0	31	0

RESULTS

Selection on phleomycin media gave rise to resistant fungal colonies only when *A. tumefaciens* LBA1100 or AGL-1 carrying the binary vector pBIN19-17 and AS were included in the co-cultivation medium. The efficiency of transformation was between 30 and 41 % in each of the three independent experiments for both *A. tumefanciens* LBA 1100 and AGL-1 (Table 1).

After five rounds of selection (2-3 weeks per round) on phleomycin putative transgenic fungi were selected at random (20 independent clones per each *A. tumefaciens* strain used) and DNA was isolated. PCR-analysis with primers corresponding to the *gpd*-promoter and the *Shble* coding sequences (20) produced the expected band (confirmed by sequencing) in all the clones analysed, which was never detected with untransformed *P. microcarpus* DNA as template (Figure 1A). Moreover, transformed fungal DNA was proved to be free of *A. tumefaciens* DNA as these samples were always negative in a PCR targeted to kanamycin resistance cassette which is located in the pBIN19-17 backbone, outside of the T-DNA (Fig. 1B). On the other hand, non-transformed *P. microcarpus* DNA was amenable to being amplified when a PCR targeted to ribosomal ITS was carried out. The fate of the transferred T-DNA (i.e. episomal, integration as a single or multiple copy) is currently under study.

Culture synthesis with *E. globulus* seedlings determined that there was no difference in ectomycorrhizal development (morphology), timing of colonisation and number of ectomycorrhizas between the transformants and wild-type *P. microcarpus* (data not shown).

DISCUSSION

For selection of fungal transformants we used the phleomycin resistance coding sequence fused to the transcription-control (promoter and terminator) sequences of the *gpd* gene from the homobasidiomycete *Schizophyllum commune*. This gene has already been shown to be efficiently transcribed and translated in other homobasidiomycetous ectomycorrhizal fungi (20).

Since *P. microcarp*us basidiospores are not easily available and protoplast are difficult and time consuming to obtain we set up an *Agrobacterium*-based transformation procedure of mycelium for *P. microcarpus* which has already been proved fruitful for genetic transformation of other ectomycorrhizal fungi (11, 20).

Herein we report the T-DNA transfer from *A. tumefaciens* to the mycelium of *P. microcarpus* for the first time. The transformation was absolutely dependent on the presence of AS in the co-culture medium indicating that the induction of *vir* genes through phenolics is essential for T-DNA transfer. In addition, its efficiency was similar to other ectomycorrhizal fungi (11, 20). There was no difference in ectomycorrhizal formation between

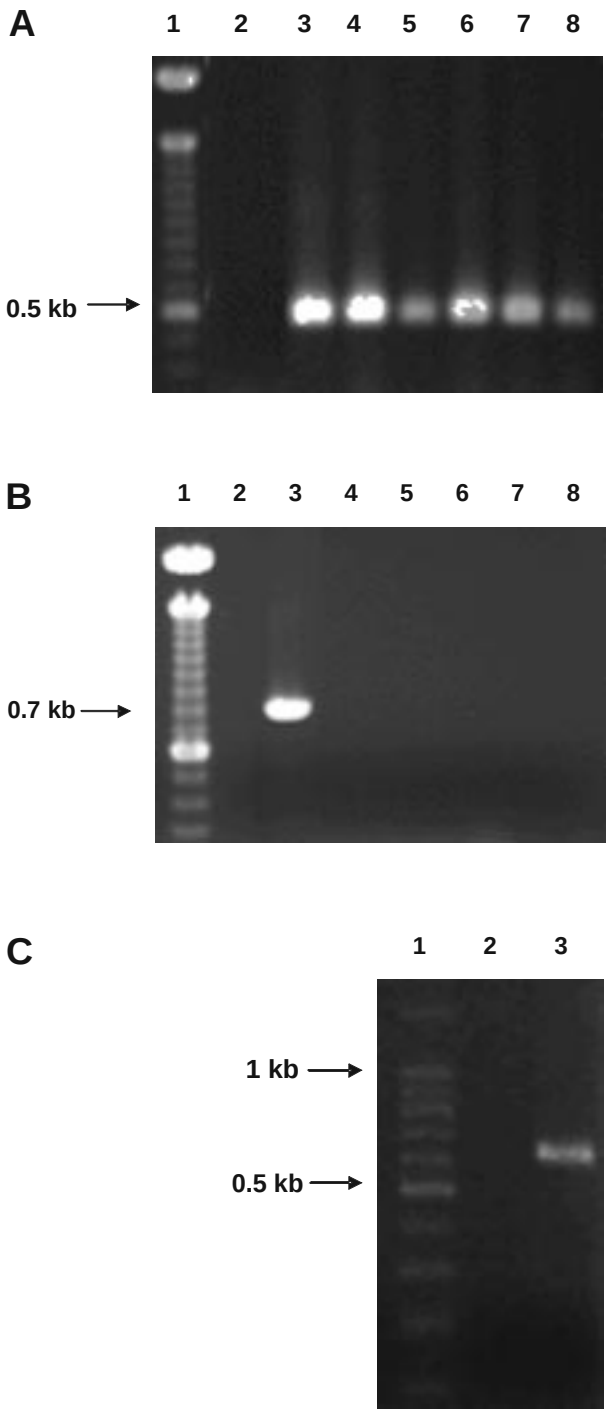

Figure 1. Polymerase chain reaction analysis of *Pisolithus microcarpus*. **(A-B).** PCR of *P. microcarpus* transformants (*lanes 4-8),* wild type (*lane 2*) and positive control with vector pBIN19-17 (*lane 3*). PCR analysis was carried out using primers GPDsen and BLEant defining a 0.5 kb transgene fragment **(A)**, and primers Kan-F and Kan-R amplifying a 0.7 kb fragment corresponding to the kanamycin resistance gene in pBIN19-17 backbone **(B)**. *Lane 1*, 100 bp ladder; *Lanes 4-5,* DNA isolated from *P. microcarpus* transformed with *A. tumefaciens* LBA 1100 (pBIN19-17). *Lanes 6-8* DNA isolated from *P. microcarpus* transformed with *A. tumefaciens* AGL-1(pBIN19-17). **(C).** PCR targeted to the fungal ribosomal ITS. *Lane 1*, 100 bp ladder; Lane 2, negative control without template; Lane 3, *P. microcarpus* wild-type. For details see materials and methods.

transformants and wild-type *P. microcarpus*. This indicates that the presence of the new phenotype did not modify the mycorrhization efficiency of the transformed strains. The *Agrobacterium*-mediated gene transfer could be a useful tool for RNA silencing (13) studies in ectomycorrhiza in order to demonstrate the role of the different *P. microcarpus* regulated genes during symbiosis formation.

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