Genetic transformation of the symbiotic basidiomycete fungus

*Hebeloma cylindrosporum*

Roland Marmeisse 1,2*, Gilles Gay 2, Jean-Claude Debaud 2, and Lorna A. Casselton 1**

1 School of Biological Sciences, Queen Mary and Westfield College, Mile End Road, London E1 4NS, UK
2 Laboratoire CNRS d’Ecologie Microbienne du Sol, Université Lyon 1, Bât. 405, 43 Boulevard du 11 Novembre, F-69622 Villeurbanne Cedex, France

Received November 22, 1991/January 10, 1992

Summary. The pAN7.1 plasmid containing the *E. coli* hygromycin B phosphotransferase gene was used to transform protoplasts of the ectomycorrhizal fungus *Hebeloma cylindrosporum*. Hygromycin-resistant transformants were selected at a frequency of one to five per µg of transforming DNA. Southern blot analyses revealed multiple copy integration of the transforming plasmid into the genome. The selection system was used to introduce other genes of interest by co-transformation. Two plasmids, one containing tryptophan biosynthesis genes and the other the NADP-glutamate dehydrogenase gene from the saprophytic basidiomycete *Coprinus cinereus*, were successfully introduced into the *H. cylindrosporum* genome with up to 70% efficiency of co-transformation. The hygromycin resistance phenotype was stably maintained during growth of transformants on hygromycin-free medium. All transformants retained their ability to form mycorrhizae with the habitual host plant *Pinus pinaster*, making them suitable for future physiological studies.

Key words: Transformation – Hygromycin – Mycorrhizal fungi – *Hebeloma*

Introduction

Mycorrhizal fungi have been extensively studied for their ability to improve host plant growth by providing mineral soil nutrients (Harley and Smith 1983). Ectomycorrhizae are highly differentiated plant organs characterized by a close contact between the fungal hyphae and the root cortical cells which possibly facilitates the exchange of metabolites between the two partners. The formation of these structures is associated with morphological and physiological changes which could be triggered at least in part by fungal phytohormones (Slankis 1973) and which might require differential gene expression (Hilbert and Martin 1988). For a better understanding of the fungal metabolic processes involved in the morphological, nutritional and molecular changes occurring during ectomycorrhiza formation and functioning, it would be of interest to specifically modify some of the biochemical pathways involved. As a first step, mutants impaired in nitrate assimilation have been isolated in our model species *Hebeloma cylindrosporum* (Wagner 1988). We now wish to develop recombinant DNA techniques to manipulate the genome and for this purpose need to establish a DNA-mediated transformation system. This will allow us to introduce multiple copies of genes of interest and to select strains with increased levels of enzyme activity.

A genetic transformation system for the ectomycorrhizal basidiomycete *Laccaria laccata* using the pAN7.1 plasmid conferring hygromycin B resistance has been developed recently (Barrett et al. 1990). In this paper we report the transformation of *H. cylindrosporum* using the same selectable marker and its use to introduce by cotransformation additional genes though to be involved either in nitrogen nutrition (the glutamate dehydrogenase gene) or in indole-3-acetic acid production (tryptophan genes). *H. cylindrosporum* is an agaric species found naturally associated with *Pinus pinaster*. Like many other ectomycorrhizal fungi this species can form mycorrhizae with several other gymnosperm and angiosperm species both in the laboratory and in the field (Valjalo 1979; Bruchet 1980; Le Tacon and Valdenaire 1980). By contrast to many other symbiotic fungi, this species can easily be manipulated in the laboratory; it has a bifactorial mating system and sporophores can be obtained under axenic conditions allowing genetic studies to be performed (Debaud et al. 1986; Debaud and Gay 1987). In addition, several protocols for producing viable protoplasts from homo- and dikaryotic strains of this species have been published (Hébraud and Fèvre 1987; Barrett et al. 1989).
Materials and methods

Strain and culture conditions. The homokaryotic strain h1 of H. cylindrosporum (Debaud and Gay 1987) was used. Cultures were grown on yeast malt glucose medium (YMG, Rao and Niederprum 1969) at 25°C. For DNA extraction the mycelium was grown on solid medium overlaid with a cellophane membrane. For protoplast preparation, 50 ml of a 1-week old liquid culture was macerated for 30 s in a Waring blender and 10 ml of macerate was used to inoculate 50 ml of liquid medium in a 250 ml conical flask. The cultures were shaken at 100 rpm and harvested after 16–24 h. Mycorhizal syntheses were performed with P. pinaster seedlings using the paper sandwich method (Chilvers et al. 1986) in Petri dishes according to Malajczuk et al. (1989).

Transformation. Protoplasts were produced by digesting mycelial cell walls at 30°C in 0.6 M mannitol containing per ml: 20 mg of cellulase Onozuka (Yakult Honsha, Japan), 5 mg of Driselase (Sigma, Pode, U.K.) and 1 mg of chitinase (Sigma). Following removal of the cell debris by filtration through glass wool, the protoplasts were washed twice in 0.6 M mannitol, once in 0.6 M mannitol, 25 mM CaCl₂ and resuspended in this solution to give a suspension containing a higher concentration of antibiotic. Mycelia growing through this second layer were isolated after 2 weeks. Details of the surface of the medium was overpoured with the same medium containing a range of antibiotic concentrations used are given in the results section. Mycelium was grown on solid medium overlaid with a cellophane membrane. For protoplast preparation, 50 ml of a 1-week old liquid culture was macerated for 30 s in a Waring blender and 10 ml of macerate was used to inoculate 50 ml of liquid medium in a 250 ml conical flask. The cultures were shaken at 100 rpm and harvested after 16–24 h. Mycorhizal syntheses were performed with P. pinaster seedlings using the paper sandwich method (Chilvers et al. 1986) in Petri dishes according to Malajczuk et al. (1989).

DNA manipulations. Standard DNA techniques were used as described by Sambrook et al. (1989). The plasmids employed in transformation experiments were purified by banding in a CsCl gradient. Genomic DNAs were extracted using the miniprep method of Zolan and Pukkila (1986). Following restriction-enzyme digests, DNA fragments were separated in 0.7% agarose gels. Southern transfer formation experiments were purified by banding in a CsCl gradient. Genomic DNAs were extracted using the miniprep method of Zolan and Pukkila (1986). Following restriction-enzyme digests, DNA fragments were separated in 0.7% agarose gels. Southern transfer was digested with 2 ml of STC (1 M sorbitol, 25 mM CaCl₂, 10 mM Tris-HCl pH 7.5) and 0.5 ml aliquots were then plated onto a selective regeneration medium (0.6 M sucrose-YMG medium with hygromycin B). After a 6-day incubation period, to allow regeneration of the protoplasts, the surface of the medium was overpoured with the same medium containing a higher concentration of antibiotic. Mycelia growing through this second layer were isolated after 2 weeks. Details of the hygromycin concentrations used are given in the results section.

Plasmids. The pAN7.1 plasmid containing the E. coli hygromycin phosphotransferase gene (hph) fused to the Aspergillus nidulans gpdA promoter and trpC terminator sequences has been described by Punt et al. (1987). The plasmid pDB03 (Burrows and Casselton, unpublished data) contains two genes of the tryptophan pathway from Coprinus cinereus. The trp-1 gene codes for tryptophan synthetase and the trp-3' promoter genes for a feedback-resistance anthranilate synthase conferring 5-fluorindole resistance in C. cinereus (Veal and Casselton 1985). The cosmids pCRM2 contains the functional NADP-specific glutamate dehydrogenase gene from C. cinereus (Marmeisse et al., unpublished data) isolated from the cosmids library described by Mutasa et al. (1990).

Results

Transformation

For each transformation experiment 8 µg of pAN7.1 was presented to at least 4 × 10⁷ total protoplasts. Transformation mixes were plated onto media containing hygromycin concentrations ranging from 25 to 100 µg x ml⁻¹. Although protoplast regeneration had initially been shown to be inhibited by 25 µg x ml⁻¹ of antibiotic, most of the viable protoplasts were regenerating on all antibiotic concentrations after 6 days. The problem was overcome by the addition of an extra layer of medium with hygromycin concentrations ranging from 50 to 150 µg x ml⁻¹ and allowed us to select genuine-resistant colonies after a further 2-weeks incubation. These hygromycin-resistant colonies were recovered at a frequency of one to five per µg of transforming DNA depending on the experiment. The selection technique worked equally well with all concentrations of hygromycin tested in both layers of medium. In control experiments, where no DNA was added to the protoplasts, no resistant colonies were selected.

Molecular analyses and level of resistance of the transformants

The genomic DNAs of 31 of these putative transformants were digested with BamH1, which cuts once in pAN7.1, and subjected to Southern blot analysis using the whole transforming plasmid as the hybridisation probe. Hybridisation signals were picked up in all cases indicating that all were true transformants with the transforming DNA integrated into their genomes. This analysis allows us to determine whether or not there are multiple copies of transforming DNA in the transformants. Where only two hybridisation bands are evident, this indicates the presence of a single integrated copy of the plasmid. However, all of the 31 transformants examined had multiple hybridisation bands. In 13 of these, a very strongly hybridising band of the same size as the linearised plasmid could be seen indicating the integration of several tandemly duplicated copies of the plasmid (see A9 and A21, Fig. 1). The multiple hybridisation bands seen in other transformants (e.g., A18 and B22, Fig. 1) could indicate separate integration events or rearrangements.

The level of resistance of individual transformants to hygromycin was studied by inoculating each onto YMG medium containing a range of antibiotic concentrations. The results of this analysis for four transformants is illustrated in Fig. 2. The growth of the untransformed strain is fully inhibited at concentrations higher than 50 µg x ml⁻¹ whereas the growth of transformant A21 remained almost unaffected by concentrations ranging from 0 to 150 µg x ml⁻¹. Transformants A18 and A21, which appear to have very different amounts of transforming DNA (Fig. 1), displayed almost similar levels of resistance suggesting that there is no correlation between plasmid copy number and expression of the resistance phenotype.

Co-transformations

We have successfully introduced two different plasmids (pDB03 and pCRM2) into the Hebeloma genome by co-