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Genetic transformation of the symbiotic basidiomycete fungus *Hebeloma cylindrosporum*

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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 co-transformation additional genes though to be involved either in nitrogen nutrition (the glutamate dehydrogenase gene) or in indole-3-acetic acid production (tryptophan genes). *H. cyclindrosporum* 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 di-karyotic strains of this species have been published (Hébraud and Fèvre 1987; Barrett et al. 1989).

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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 Niederpruem 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 blendor 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. Mycorrhizal 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 $CaCl_2$ and resuspended in this solution to give a suspension of $2-7 \times 10^7$ protoplasts per 100 µl. Transformations were essentially carried out as described by Casselton and de la Fuente Herce (1989). To 100 µl of protoplast suspension were added 20 µl of DNA in TE buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA) and 25 µl of PEG solution (25% polyethylene glycol 4000, 25 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5). After a 20 min incubation on ice, an additional 1 ml of PEG solution was added followed by a 5-min incubation at room temperature. The solution was diluted with 2 ml of STC (1 M sorbitol, 25 mM $CaCl_2$, 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.

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 and DNA labelling were carried out as described by Mellon et al. (1987) using Hybond N membrane (Amersham, UK) and nick translation kit from BRL (Maryland, USA). Hybridisations and post-hybridisation washes were at 65 °C.

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*^{iar} gene codes for a feedback-resistance anthranilate synthase conferring 5-fluoroindole resistance in *C. cinereus* (Veal and Casselton 1985). The cosmid pCRM2 contains the functional NADP-specific glutamate dehydrogenase gene from *C. cinereus* (Marmeisse et al., unpublished data) isolated from the cosmid library described by Mutasa et al. (1990).

Results

Transformation

For each transformation experiment 8 µg of pAN7.1 was presented to at least 4×10^7 total protoplasts. Transformation mixes were plated onto media containing hygromycin concentrations ranging from 25 to 100 µg × ml^{-1}. Although protoplast regeneration had initially been shown to be inhibited by 25 µg × 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 × 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 *Bam*H1, 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 × ml^{-1} whereas the growth of transformant A21 remained almost unaffected by concentrations ranging from 0 to 150 µg × 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-

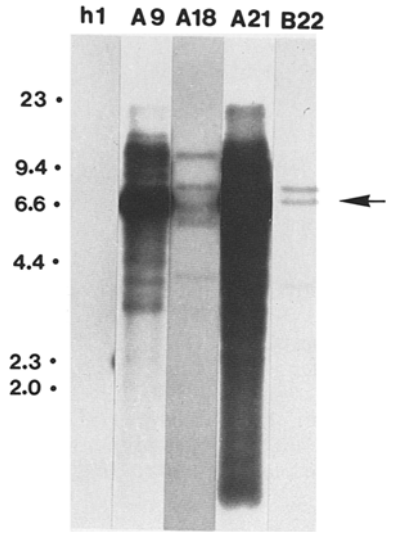

Fig. 1. Southern blot analysis of *Bam*HI-digested genomic DNA of *H. cylindrosporum* transformants probed with ^{32}P-labelled pAN7.1. *h1*, untransformed control; *A9*, *A18*, *A21*, *B22*, four representative transformants. A lambda *Hin*dIII digest was used as a DNA size marker. *Arrow*, size of the linearised pAN7.1 plasmid

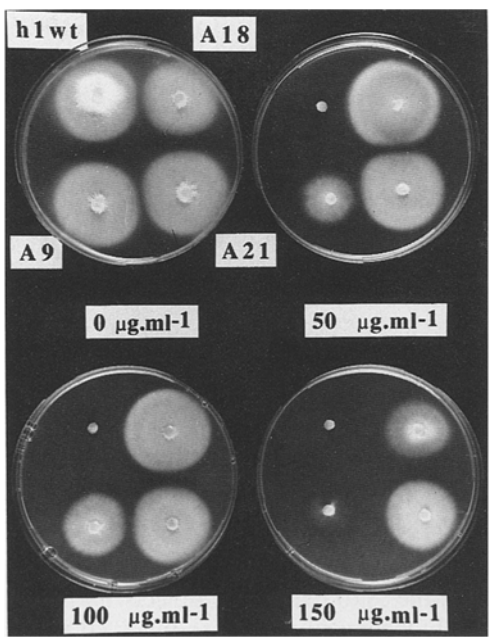

Fig. 2. Growth of the untransformed control (*h1*) and of three transformants containing the integrated pAN7.1 plasmid (*A9*, *A18*, *A21*) on varying concentrations of hygromycin in YMG medium. Picture taken 15 days after inoculation

transforming with pAN7.1 and selecting for hygromycin-resistant transformants. For each transformation 4 µg of pAN7.1 and 13 µg of pDB03 or 7 µg of pCRM2 was used. Hygromycin-resistant colonies were recovered in both cases. Their genomic DNAs were analysed for the presence of the co-transformed plasmid using hybridisation probes specific to pDB03 and pCRM2 (Fig. 3). Neither probe hybridised to the DNA of the untransformed strain but both hybridised to the DNA of appropriate transformants. From experiments with pDB03 (Fig. 3, transformants C3 to D2), ten of 14 transformants analysed had the *trp-3* gene, which corresponds to a 70% co-transformation efficiency. The hybridisation patterns indicated that there could be several copies of the gene. *Hin*dIII, used to digest the genomic DNA, cuts out the *trp-3* gene from plasmid pDB03 on a 4.8 kb fragment. A multiple banding pattern obtained with the gene as a hybridisation probe means that several different integration events may have occurred within this fragment or else that there have been rearrangements. The *trp-3^{iar}* gene present in pDB03 confers 5-fluoroindole (5-FI) resistance in *C. cinereus* (Veal and Casselton 1985). It was, therefore, possible to test for expression of this gene in *H. cylindrosporum* transformants. Of the ten transformants shown to contain *trp-3^{iar}*, C3, C6, C9 and C15 were found to be resistant to 1×10^{-5} M 5-FI, a concentration which severely inhibited growth of the untransformed control.

For the transformation with pCRM2, hybridisation signals were detected in three of the four transformants analysed (Fig. 3, transformants C19, C20 and D11). However, only transformant C20 gave a hybridisation pattern consistent with having the entire pCRM2 integrated. The two other co-transformants (C19 and D11) gave very weak hybridisation signals with a few hybridising bands visible (Fig. 3). They appeared to have lost most of the pCRM2 sequences prior to, or following, integration into the genome. Hybridisation between pAN7.1 and pCRM2 vector sequences can be ruled out since these are not homologous. The vector in pCRM2 is derived from lambda and is the cosmid Lorist 2 (Cross and Little 1986), whereas the vector in pAN7.1 is the plasmid pUC18 (Punt et al. 1987).

Mitotic stability of the transforming DNA and ability to form mycorrhizae

To see if the transforming DNA was stably integrated into the genome during vegetative growth, all transformants were grown for two successive 3-week periods on solid YMG medium without hygromycin before being transferred back to YMG containing different concentrations of hygromycin. Growth without selection pressure did not affect the level of hygromycin resistance of any of the transformants analysed.

The transformants were successfully mated to a sexually compatible homokaryon to give dikaryotic mycelia. All these different transformed homokaryons (39 including the different co-transformants) and the corresponding dikaryons were used to inoculate the root system of *P. pinaster* seedlings. Short dichotomous mycorrhizae were observed in all cases 3–4 weeks after inoculation. No significant morphological difference was detected between mycorrhizae formed by the transformants and the wild-type mycelia. Excised mycorrhizae were transferred onto hygromycin-containing medium and all gave rise to a resistant mycelium showing that the transforming DNA had not been lost.

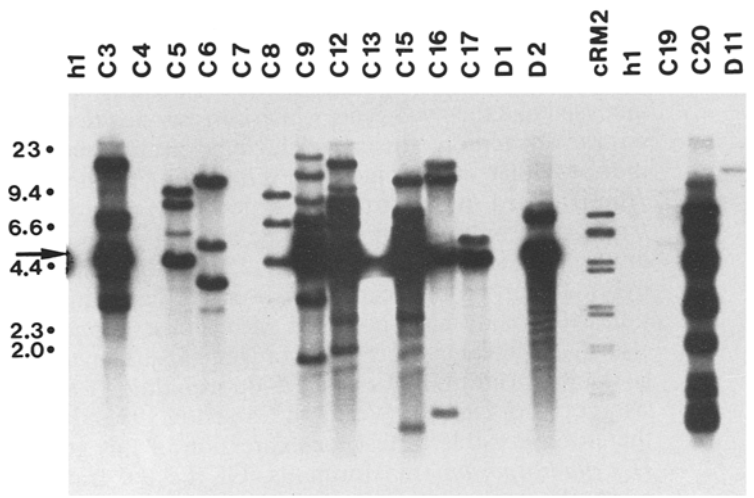

Fig. 3. Southern blot analysis of genomic DNAs of *H. cylindrosporum* strains co-transformed with pAN7.1 and pDB03 (*C3 to D2*) or pCRM2 (*C19*, *C20* and *D11*). DNAs were digested with *Hin*dIII and probed with the ^{32}P-labelled *C. cinereus trp-3* gene (isolated as a 4.8 kb *Hin*dIII fragment of pDB03: *arrow*) (pDB03 co-transformants) or pCRM2 (pCRM2 co-transformants). *h1*, untransformed control; *CRM2*. *Hin*dIII digest of the plasmid

Discussion

We have demonstrated that wild-type mycelia of *H. cylindrosporum* can be stably transformed to hygromycin resistance using the pAN7.1 plasmid. The transformation frequencies obtained (one to five transformants per μg of DNA) were low when compared to many other transformation systems developed for saprophytic and parasitic fungal species (Fincham 1989), and all of the transformants analysed had integrated several copies of the transforming plasmid. It is very likely that the hygromycin resistance gene is poorly transcribed in *H. cylindrosporum* and many copies of the gene need to be present in order to give the resistance phenotype. An important factor that may affect the level of resistance is the site of integration of the transforming DNA. For *C. cinereus* Mellon and Casselton (1988) clearly demonstrated that copy number and gene expression are not correlated. Furthermore, the *A. nidulans gpdA* (encoding the glyceraldehyde-3-phosphate dehydrogenase) promoter in pAN7.1 is unlikely to act as a strong promoter in such a distantly related host. It has been shown previously that some genes from *A. nidulans* are not expressed in the basidiomycete *C. cinereus* (Casselton and de la Fuente Herce 1989). To increase our transformation frequencies, it might be useful to characterise a basidiomycete promoter from *H. cylindrosporum* to ensure better expression of the *hph* gene in this fungus. Mooibroek et al. (1990) have demonstrated that in another basidiomycete, *Schizophyllum commune*, there is extensive methylation of the pAN7.1 plasmid following transformation, which could also account for the low level of hygromycin resistance in the transformants.

The two plasmids used for co-transformation experiments carry genes whose activities can be related to the formation and functions of mycorrhizae. The first one, pDB03, contains two genes of the trypotphan pathway; this amino acid is the required precursor of auxin compounds produced by mycorrhizal fungi and which are thought to play a role in the infection process (Slankis 1973). The second one, pCRM2, contains the NADP-glutamate dehydrogenase gene; this enzyme plays a role in ammonium assimilation by the fungus and could, therefore, be implicated in the improvement of the nitrogen nutrition of the host plant (Martin and Botton 1991). These two plasmids were successfully introduced into the *Hebeloma* genome though it appears that it will be necessary to subclone the *gdh* gene as a small fragment to ensure that it is not lost from the large cosmid insert. Our preliminary data on 5-fluoroindole resistance with pDB03 co-transformants suggest that the tryptophan genes from *C. cinereus* are expressed in at least some transformants. We have been unable to use pDB03 to select directly for transformants resistant to 5-FI but this may simply be a technical problem of selection.

The resistance phenotype of the *H. cylindrosporum* transformants is neither lost after a growth period on unselective medium nor after infection of the host plant. The stability of the transforming DNA and the ability of the transformants to form mycorrhizae are two necessary conditions for the use of these mycelia in physiological experiments.

The transformation system based on hygromycin resistance should enable us to realise our objective which is to modify fungal biochemical pathways involved in the symbiotic processes. Despite its relatively low efficiency, this system presents several advantages. Firstly, wild-type strains can be directly transformed without the need to select auxotrophic mutants, which can be laborious particularly in an organism lacking asexual spores and in which mutagenesis procedures could interfere with essential symbiotic functions. Secondly, the co-transformation frequency is high enough to enable us to select, even in a small sample of transformants, co-transformants, with multiple copies of the co-transforming plasmid.

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