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Short Communication

Agrobacterium tumefaciens-mediated transformation of *Paracoccidioides brasiliensis*

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Paracoccidioides brasiliensis is the causative agent of paracoccidioidomycosis, an important mycosis endemic to Latin America. As the tools to study gene function in *P. brasiliensis* are only in the early stage of development, there is presently no system that allows for both the delivery and integration of exogenous nucleic acids into its genome. We report in this paper the transformation of the yeast phase of *P. brasiliensis* (ATCC-60855) with *Agrobacterium tumefaciens* (GV3101) carrying the vector pAD1625. The microorganisms were co-cultivated for 2 days and then incubated for 10 days at 35°C on selective media. PCR and dot-blot targeted at a fragment of 222 bp from the *hph* (hygromycin phosphotransferase) gene which confers Hyg^r confirmed the transformation of *P. brasiliensis.*

Keywords *A. tumefaciens*, *P. brasiliensis*, transformation

Introduction

The dimorphic fungus *Paracoccidioides brasiliensis* is the etiologic agent of paracoccidioidomycosis, one of the most prevalent endemic mycoses in Latin America [1]. Studies in experimental models have demonstrated that once the conidia are inhaled into the lungs, they are transformed into the yeast phase, which may then disseminate to other visceral organs [2]. Little is known of the pathogenic processes that underline this sequence of events. An advanced genetic procedure for mutagenesis and phenotypic screening would be valuable in identifying virulence factors or factors involved in regulating other processes (e.g. phase transition). This could be a powerful approach when mutagenesis involves random insertions of a known DNA sequence into the genome [3].

One highly successful insertional mutagenesis method in plants has made use of the transfer DNA (T-DNA) of the plant pathogen, *Agrobacterium tumefaciens*. *A. tumefaciens* carries a ~200-kb tumor-inducing (Ti) plasmid, within which is a portion referred to as T-DNA that is randomly inserted into the plant genome [4]. The Ti plasmid has been modified extensively, its tumor-causing genes and other superfluous genetic components have been removed and binary vectors have been constructed that are able to replicate, upon addition of T-DNA selectable markers, in *Escherichia coli* as well as in fungi [5].

While Soares *et al*. did describe the use of electroporation in a poster at a scientific meeting [6], there is no specific method in the literature for the transformation of *P. brasiliensis*. Therefore, we present in this report a practical and convenient transformation system for *P. brasiliensis* using *A. tumefaciens.*

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Materials and methods

Strains

Paracoccidioides brasiliensis strain ATCC-60855 was obtained from the Corporacion para Investigaciones Biologicas (CIB) Medellín, Colombia, culture collection and maintained in the yeast phase at 36°C on brain–heart infusion (BHI) agar slants supplemented with glucose at 1%. *A. tumefaciens* strains GV3101 and LBA4404 were obtained from R. Arango (Vegetal Biotechnology Group, CIB; see Table 1 for further information).

Plasmids

The vectors pAD1625 and pAD1624 were kindly donated by John N. Galgiani (Department of Internal Medicine, College of Medicine, University of Arizona, Tucson, AZ) [7]. These plasmids contained the *E. coli* hygromycin B phosphotransferase (*hph*) gene (encoding hygromycin phosphotransferase, conferring Hyg^r), the wide host-range replicon 75Δ2 and the constitutive *vir* gene mutant *vir*GN54D. However, the two vectors differ as to the promoter sequence that drives the *hph* gene (i.e. *Neurospora crassa cpc1* in pAD 1625 and *Aspergillus nidulans trpC* in pAD 1624).

The plasmids were introduced into *A. tumefaciens* GV3101 and LBA4404 by electroporation (Gene Pulser; Bio-Rad, Richmond, CA) [8] and transformants were isolated by ampicillin and tetracycline selection at 100 and 15 μg/ml, respectively.

Fungal transformation

Paracoccidioides brasiliensis yeast suspensions were prepared for transformation by inoculating a portion of the growth of a fresh (5-day-old) BHI agar slant into 50 ml BHI broth supplemented with glucose at 1%. The cells were grown for 72 h at 36°C with shaking, then collected by centrifugation at 4500 *g* for 10 min and re-suspended in 10 ml induction medium (BHI glucose 1%, 25 mmol/l 2:[N- morpholino] ethanesulfonic acid {MES}, pH 5.8. The culture was then diluted to 1×10^7 yeasts per ml media.

Table 1 Strains used

Strain	Description
Agrobacterium tumefaciens LBA4404	Ach5 strain containing pAL4404 an octopine-type plasmid, harboring a T-DNA deletion but having a full set of *vir* gene [18,19]
GV3101	This strain contains the Ti-plasmid pMP90 [20]
Paracoccidioides brasiliensis ATCC 60855	Strain obtained from a Colombian patient with chronic multifocal paracoccidioidomycosis [21]

Both isolates of *A. tumefaciens* (LBA4404 and GV3101) were grown for 48 h in 2 ml Luria Bertani (LB) liquid medium [9] containing ampicillin (100 μg/ml) and tetracycline (15 μg/ml) at 28°C on a shaker at 150 r.p.m. Once an optical density of 1.0–1.5 at A_{600} was attained, 1 ml was removed and added to 50 ml induction medium. These cultures were grown as indicated above until a density of 0.5–1.0 at A_{600} was achieved. The bacteria were then collected by centrifugation at 4500 *g* for 10 min, washed once and resuspended in 10ml induction medium plus ampicillin and tetracycline to obtain a final concentration of 1×10^9 cells.

For co-cultivation, yeast phase cells of *P. brasiliensis* were mixed with *A. tumefaciens* cells at varying ratios (1:10, 1:5, 1:1, 1:0.5, 1:0.1, 1:0.01) in sterile microfuge tubes. The cell mixtures were inoculated onto sterile 0.45-μm nitrocellulose filters, which in turn were placed on solid induction medium plus ampicillin and tetracycline and incubated for 2 days at 28°C. After co-cultivation, filters were washed with 1 ml of a normal saline solution containing hygromycin B (100 μg/ml). Cells were dislodged by vortexing, plated on BHI agar containing both 8 μg/ml gentamicin and 200 μmol/l cefotaxime for selection of *A. tumefaciens* and 100 μg/ml hygromycin for selection of *P. brasiliensis* transformants. Plates were incubated for 10 days at 35.5°C and the fungal colonies that developed were sub-cultured for further studies. All experiments included negative controls (i.e. yeast cells co-cultivated with plasmid free *A. tumefaciens* cells).

Transformant polymerase chain reaction

Genomic DNA was obtained from the putative transformants to be tested for the presence of the *hph* gene through the use of polymerase chain reaction (PCR). For *hph* PCR, a 222-bp fragment was amplified by using 100 ng template DNA, 0.2 mmol/l of each dNTP, 0.2 pmol of each primer, *hph*1 5′–GTGTCACGTTGCAAGACCTGCC 3′ and primer *hph*2 5′–TGACGGTGTCGTCCATCACAGT 3′ (Operon, Alameda, CA), one unit of Taq DNA polymerase, 1 × reaction buffer containing 1.0 mmol/l $MgCl_2$ (Promega, Madison, WI) in a final volume of 50 μl. The following PCR cycling temperatures were used: 95°C for 5 min, 35 cycles of 30 s each at 95, 65 and 72°C and finally one cycle at 72°C for 7 min. Controls consisted of a PCR using primers specific for

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the 16s ribosomal gene or for a *P. brasiliensis* specific gene [10].

Dot-blot hybridization

Dot-blot analyses were performed with genomic DNA from both the putative transformants and the untransformed *P. brasiliensis* using standard protocols [9]. The probe for the transforming DNA was a 222-bp *hph* fragment prepared by PCR as described above. The PCR product was labeled with alkaline phosphatase using the AlkPhos Direct kit from Amersham Pharmacia Biotech (Piscataway, NJ).

Results

Paracoccidioides brasiliensis *transformation mediated by* A. tumefaciens

Agrobacterium tumefaciens GV3101 was transformed with both pAD1625 and pAD1624 and was subsequently employed at several ratios in co-cultivation with *P. brasiliensis* for 24 and 48 h. Every experiment was done at least twice. Only the *A. tumefaciens* GV3101 transformed with the pAD1625 yielded transformants when co-cultivated for 48 h at a ratio of 1.0 : 0.5 (fungi : bacteria). This provided 33 and 35 colonies, equivalent approximately to 3.4 transformants per 10^6 target yeast cells. The other ratios and times employed gave no colonies or very few of them (between one and five). The colonies obtained were subcultured to solid medium containing hygromycin 100–200 μg/ml with approximately 75% of the putative transformants revealing resistance to these concentrations (Fig. 1). All experiments were conducted twice and spontaneous resistance to hygromycin was not observed.

Mitotic stability

Twenty-two of the transformants were tested for stability by growing them on BHI agar, without hygromycin, for 45 days, subculturing to fresh medium every 5 days. Portions of these colonies were then transferred to BHI agar containing 100, 150 and 200 μg/ml hygromycin for 1 week. All the transformants showed the same level or resistance, with growth equal to that of the control non-transformed fungi.

Characterization of transformant isolates

Polymerase chain reaction was used to determine whether or not the *hph* gene was present in the DNA of the 22 stable transformants and revealed a band of 222 bp specific for *hph* in all the transformants, but not in the controls. As a positive PCR control, the GP43 gene was amplified giving a band of 533 bp. Figure 2a shows several colonies representing amplification of the *hph* and the GP43 genes.

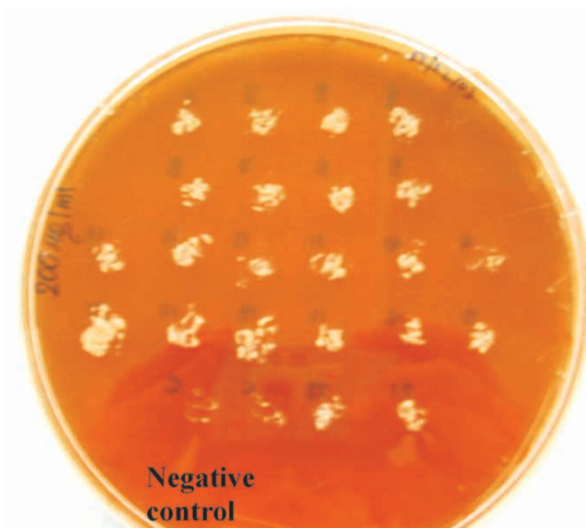

Fig. 1 *Paracoccidioides brasiliensis* yeast transformed with pAD1625 plated in brain–heart infusion (BHI) medium with hygromycin 200 μg/ml. Two non-transformed control colonies, at the bottom left, did not show growth.

In order to corroborate that the PCR band corresponding to the hygromycin gene was not due to the presence of *A. tumefaciens*, PCR studies using specific primers for the bacterium gene (16s ribosomal DNA) were conducted. While the control amplicon gave a band of 662 bp, it was absent in the *P. brasiliensis* DNA from transformed colonies (data non shown). Genomic DNA was also used for dot-blot hybridization analysis, using the 222-bp amplicon as a probe. All the colonies growing on the hygromycin media gave a positive hybridization, indicating the presence of the *hph* gene in *P. brasiliensis* genome (Fig. 2b).

Discussion

Initial attempts to obtain *P. brasiliensis* transformation by electroporation using protocols shown to be successful in other dimorphic fungi (e.g. *Histoplasma capsulatum*) have failed [11]. This prompted us to investigate other approaches such as those recently published by Abuodeh *et al*. in which *Coccidioides immitis* transformation was mediated by *A. tumefaciens.* The technical simplicity and efficiency of this procedure, as well as minimum manipulation, recommended its use in our laboratory. This technique had also been used for the transformation of *Saccharomyces*

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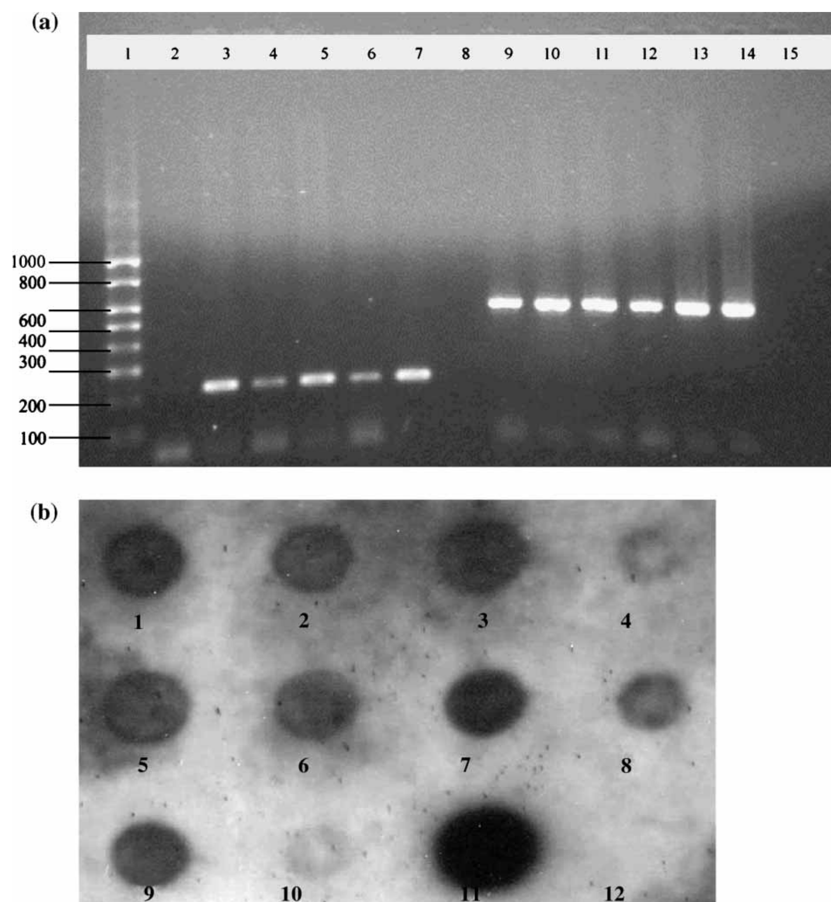

Fig. 2 (a) Polymerase chain reaction (PCR) analysis of *hph* gene and GP43 gene. Lane 1: molecular weight markers. Lane 2: negative control of untransformed *Paracoccidioides brasiliensis* tested with specific *hph* gene primers. Lines 3–7: specific band (222 bp) of *P. brasiliensis* transformants amplified with specific *hph* gene primers. Lane 8: negative control for the *hph* gene specific primers without DNA. Lane 9: untransformed *P. brasiliensis* tested with primers specific for the GP43 gene (533 bp). Lanes 10–14: correspond to species-specific band (533 bp) in *P. brasiliensis* transformants amplified with specific GP43 gene primers. Lane 15: negative control specific for the GP43 gene primers with no DNA. (b) Dot-blot analyses of seven *P. brasiliensis* transformants. Samples 1, 2, 3, 5, 6, 7 and 9 correspond to transformed *P. brasiliensis* with the *hph* gene. Samples 4, 8 and 10 correspond to negative controls (non-transformed *P. brasiliensis).* Position 12 contains no sample.

cerevisiae [12–14], filamentous fungi [15,16] and fungi of industrial importance such as *Agaricus bisporus* [17].

Employing the same plasmids as those described by Aboudeh *et al.*, we transformed *A. tumefaciens* GV3101 and managed subsequently to transform *P. brasiliensis* with the plasmid pAD1625, confirming the findings of other researchers [5,7]. As in the transformation of *C. immitis*, we employed the plasmid pAD1625, which had a constitutive mutation, expressing all the *vir* genes that improve transformation in plants [17] and in fungi, as evidenced by studies with *C. immitis* [18] and *P. brasiliensis*. In our investigations, only plasmid (pAD1625) was capable of causing this transformation. One of the advantages of *A. tumefaciens*-mediated transformation is that in most of the cases, the integration occurs at a single site of insertion [5,7]. We are currently evaluating this insertion in our transformants.

Besides constituting an appropriate method for transforming *P. brasiliensis*, the procedure could be applied to other molecular studies, such as development of a mutagenesis system by random insertion in genes that may produce changes in the morphology of the fungus. In addition, it could be used to produce specific gene mutants by means of homologous recombination, such as previously reported in *Aspergillus* spp. [16].

We are confident that the efficiency of transformation can be improved by changing some of the parameters employed in the procedure, such as time of co-cultivation, ratio between bacteria and fungi and conditions of the fungal cells.

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