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Genetic manipulation of *Fonsecaea pedrosoi* using particles bombardment and *Agrobacterium* mediated transformation

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ABSTRACT

Fonsecaea pedrosoi, a melanized fungal pathogen that causes Chromoblastomycosis, a human disease with a worldwide distribution. Biolistic is a widely used technique for direct delivery of genetic material into intact cells by particles bombardment. Another well-established transformation method is *Agrobacterium*-mediated transformation (ATMT), which involves the transfer of a T-DNA from the bacterium to the target cells. In *F. pedrosoi* there are no reports of established protocols for genetic transformation, which require optimization of physical and biological parameters. In this work, intact conidia of *F. pedrosoi* were particle bombarded and subjected to ATMT. In addition, we proposed hygromycin B, nourseothricin and neomycin as dominant selective markers for *F. pedrosoi* and vectors were constructed. We tested two parameters for biolistic: the distance of the particles to the target cells and time of cells recovery in nonselective medium. The biolistic efficiency was 37 transformants/µg of pFpHYG, and 45 transformants/µg of pAN7.1. Transformants expressing GFP were successfully obtained by biolistic. A co-culture ratio of 10: 1 (bacterium: conidia) and co-incubation time of 72 h yielded the largest number of transformants after ATMT. Southern blot analysis showed the number of foreign DNA insertion into the genome is dependent upon the plasmid used to generate the mutants. This work describes for the first time two efficient methods for genetic modification of *Fonsecaea* and these results open new avenues to better understand the biology and pathogenicity of the main causal agent of this neglected disease.

1. Introduction

Fonsecaea pedrosoi is one of the causative agents of the human disease chromoblastomycosis (CBM). This filamentous fungus produces melanin, which forms a thick layer on the cell wall (Franzen et al., 2008; Cunha et al., 2010). The fungus lifecycle includes distinct morphological stages, comprising the conidia, mycelium (infective forms) and sclerotic cells (parasitic form, also called muriform cells). *F. pedrosoi* is commonly found in tropical and subtropical areas, inhabiting plants (thorns of tropical plant *Mimosa pudica* or Babassu coconut shell) and decomposing organic materials (Marques et al., 2006; Salgado et al., 2004; Vicente et al., 2008; Vicente et al., 2013).

Chromoblastomycosis is a chronic, granulomatous and suppurative mycosis, affecting the subcutaneous tissue characterized by the appearance of varied morphology lesions as verrucous nodules, cauliflower like tumors or psoriasis like plaques (Queiroz-Telles et al., 2017a). The infection often occurs in the lower limbs when there is traumatic implementation of conidia or fragments of hyphae in the skin. *F. pedrosoi* is the main etiological agent, but the disease has others causative agents belonging to *Chaetothyriales* order (Ascomycota, *Eurotiomycetes*), including *Phialophora*, *Cladophialophora*, *Exophiala* and the *Rhinocladiella* genus (Aragão et al., 1996; de Azevedo et al., 2015; Najafzadeh et al., 2010; Naka et al., 1988; Seyedmousavi et al., 2014; Silva et al., 1998; Surash et al., 2005). CBM is an endemic neglected

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disease, which affects millions of individuals worldwide especially in Latin America, Asia and Africa. High incidence in rural workers not adequately protected during agricultural and extractivism practices, makes it as an occupational disease (Menezes et al., 2008; Queiroz-Telles et al., 2017a,b; Silva et al., 1995).

Genetic transformation is the process of controlled introduction of exogenous nucleic acids on a genome, without compromising the viability of the cells. There are several methods of gene transfer and the most common are: chemically mediated DNA transformation, electroporation and particle bombardment (He et al., 2017; Meyer, 2008; Olmedo-Monfil et al., 2004; Rivera et al., 2014). The biolistic or particle bombardment is a method that consists in the acceleration of gold or tungsten micro particles coated with exogenous DNA and directed by vacuum to cross the cell wall and membrane in a non-lethal way in different types of cells and tissues (Meyer, 2008; Rivera et al., 2014). A great advantage of this method is that it does not require pretreatment of cells and removal of the cell wall; however, it requires specific equipment and high cost supplies, besides the fact that it usually promotes the insertion of multiple copies of exogenous DNA in the genome of the target cell (Meyer, 2008; Rivera et al., 2014). Biolistic was firstly described for fungal genetic manipulation by Armaleo et al. (1990) and has been applied for a variety of species from yeasts to molds (reviewed in Rivera et al., 2014). Genetic transformation mediated by *Agrobacterium tumefaciens* (ATMT) has been employed to a wide number organisms including plant and mammalian cells (for review see Matveeva and Lutova, 2014; Kunik et al., 2001). *Agrobacterium* is a gram-negative soil bacterium that transfers a DNA (T-DNA) located in the Tumor inducing Ti-plasmid, in response to plant phenolic compounds to random sites into the genome of the target cells (Zambryski, 1988). This method has been described for several fungal species over at last two decades as an efficient and powerful tool for genetic manipulation (Abuodeh et al., 2000, Bundock et al., 1995, De Groot et al., 1998, for review Frandsen, 2011), since it requires no special equipment or cell enzymatic treatments. Despite these two techniques have been extensive successfully used for fungal research providing new insights into biology and pathogenesis, they have limitations and advantages to be considered in order to optimize the parameters that affect the genetic transformation efficiency of different fungi.

The biology, virulence factors and molecular mechanisms of pathogenicity of *F. pedrosoi* are still poorly understood. Although the genomic sequencing of *F. pedrosoi* was recently concluded (Teixeira et al., 2017), the functional genetic studies are limited in this pathogen since until now, there has been no report of molecular tools for the genetic manipulation of the main etiological agents of chromoblastomycosis. This work describes and compares two genetic transformation system for *F. pedrosoi*: the mediated by *Agrobacterium* and particle bombardment, as well as reports the construction of plasmids containing three dominant selective markers. The optimization of genetic transformation protocols for *F. pedrosoi* may aid for the understanding of chromoblastomycosis and their causative agents and contribute to the development of future strategies for management of the disease.

2. Material and methods

2.1. Strains and culture conditions

The *F. pedrosoi* strain CBS 271.37 (ATCC18658) was used for all the experiments and maintained on Sabouraud Dextrose Agar (SDA) (for 1 L: 10 g peptone, 40 g dextrose, 15 g agar pH: 5.6 autoclaved at 120 °C/15 min) at 25 °C for seven days. To obtain the conidia, *F. pedrosoi* was grown in Potato Dextrose Broth (PDB) (Difco – for 1 L: 4 g starch from potato infusion, 20 g dextrose, pH adjusted to 5.6) for seven days at 28 °C under 200 rpm shaking. The culture was vigorously vortexed for 1 min to release the conidia, filtered through sterile glass wool assembled in funnels and centrifuged at 3000 × *g* for five minutes. Conidia cells were ressuspended in saline (0.9% NaCl) and counted in Neubauer's chamber to adjust the density. *Escherichia coli* DH5α and *Agrobacterium tumefaciens* EHA105 were culture in LB medium.

2.2. Determination of F. pedrosoi susceptibility to the antibiotics used as selective dominant markers

10^3, 10^5, 10^7 of *F. pedrosoi* conidia were plated in SDA supplemented with hygromycin B (Invitrogen) or nourseothricin (Jena Bioscience) or neomycin/G418 (Invitrogen) at three different concentrations 0, 25, 50 and 100 µg/mL. Antibiotic sensitivity was estimated based on mycelial growth after incubation for up to 30 days at 25 °C.

2.3. Construction of plasmids

Genomic sequences of *F. pedrosoi* were accessed at FungiDB database (http://fungidb.org/). The hygromycin B resistance (hygR) cassette driven by *F. pedrosoi gapdh* promoter and *trpC* terminator was constructed by overlapping PCR. Both 1.1 kb and 0.34 kb related to *Fp*P*gapdh* (primers Fp6 and Fp7) and *Fp*T*trpC* terminator (primers Fp3 and Fp5), respectively were PCR amplified from the genomic DNA of CBS271.37, while pAN7.1 (Punt et al., 1987) was used as the template for *hph* (hygromycin B phosphotransferase) amplification with primers Fp1 and Fp2. To construct the final cassette, the three fragments were fused by overlapping PCR (primers Fp10 and Fp11) and the *Fp*P*gapdh*:*hph*:*Fp*T*trpC* construct was cloned into pBluescript KS + linearized with BamHI and EcoRV. The final construct was inserted into competent *E. coli* via heat shock method and selected on LB agar with 100 µg/mL ampicillin. The insertion was confirmed after BamHI and

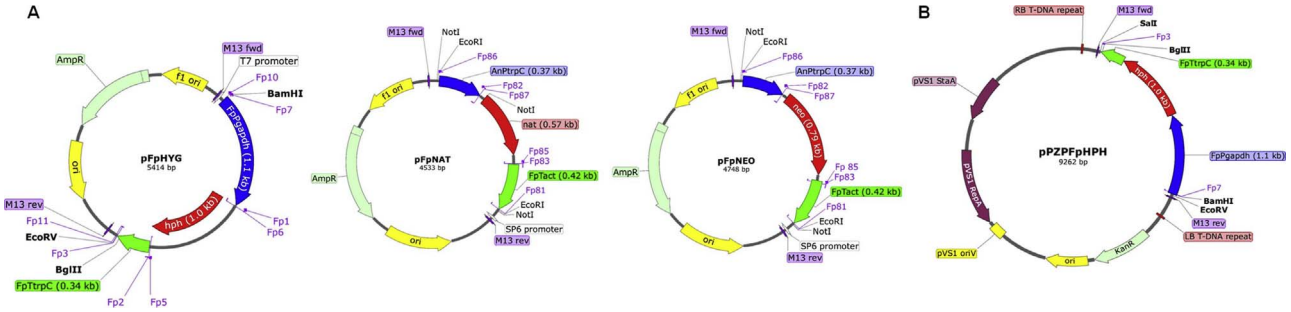

Fig. 1. Schematic representation of plasmids constructed in this work carrying resistance gene to Hygromycin B, Nourseothricin and Neomycin. (A) Plasmids used to biolistic transform *F. pedrosoi* spores: (1) pFpHYG has Hygromycin resistance cassette cloned in *BamH*I and *Eco*RV restriction sites of pBluescript KS + ; (2) pFpNAT has Nourseothricin resistance cassette cloned in *Eco*RI restriction site of pGEMT-Easy (Promega); (3) pFpNEO has Neomycin resistance cassette cloned in *Eco*RI restriction site of pGEMT-Easy (Promega) (B) Plasmid used for *Agrobacterium* mediated transformation of *F. pedrosoi* spores: pPZPFpHPH has Hygromycin resistance cassette cloned in *BamH*I and SalI restriction sites of pPZP-201BK (Walton et al., 2005). The oligonucleotides positions and the size of the fragments are indicated on the figure. Fp: *Fonsecaea pedrosoi*; An: *Aspergillus nidulans*; *trpC*: Tryptophan biosynthesis protein, *gapdh*: glyceraldehyde-3-phosphate dehydrogenase; *act*: actin; P: promoter; T: terminator.